(51) International Patent Classificati n 5 : C12N 1/21, 15/29, 15/82 C07H 15/12	**A1**	(11) International Publication Number: **WO 91/13972** (43) International Publication Date: 19 September 1991 (19.09.91)

(21) International Application Number: PCT/US91/01746

(22) International Filing Date: 14 March 1991 (14.03.91)

(30) Priority data:
494,106   16 March 1990 (16.03.90)   US
567,373   13 August 1990 (13.08.90)   US
615,784   14 November 1990 (14.11.90)   US

(60) Parent Application or Grant
    (63) Related by Continuation
    US   494,106 (CIP)
    Filed on   16 March 1990 (16.03.90)

(71) Applicant *(for all designated States except US)*: CALGENE, INC. [US/US]; 1920 Fifth Street, Davis, CA 95616 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : THOMPSON, Gregory, A. [US/US]; 5127 Cowell Blvd., Davis, CA 95616 (US). KNAUF, Vic, C. [US/US]; 1013 Hillview Lane, Winters, CA 95694 (US).

(74) Agents: LASSEN, Elizabeth et al.; Calgene, Inc., 1920 Fifth Street, Davis, CA 95616 (US).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PLANT DESATURASES - COMPOSITIONS AND USES

(57) Abstract

By this invention, compositions and methods of use of plant desaturase enzymes, especially Δ-9 desaturases, are provided. Of special interest are methods and compositions of amino acids and nucleic acid sequences related to biologically active plant desaturases as well as sequences, especially nucleic acid sequences, which are to be used as probes, vectors for transformation or cloning intermediates. Biologically active sequences may be found in a sense or anti-sense orientation as to transcriptional regulatory regions found in various constructs.

1

PLANT DESATURASES -
COMPOSITIONS AND USES

5

This application is a continuation-in-part of USSN
07/494,106 filed on March 16, 1990 and a continuation-in-
part of USSN 07/567,373 filed on August 13, 1990 and a
continuation-in-part of USSN 07/615,784 filed on November
10   14, 1990.

Technical Field

The present invention is directed to desaturase
enzymes relevant to fatty acid synthesis in plants,
15   enzymes, amino acid and nucleic acid sequences and methods
related thereto, and novel plant entities and/or oils and
methods related thereto.

INTRODUCTION

20 ## Background

Novel vegetable oils compositions and/or improved
means to obtain or manipulate fatty acid compositions, from
biosynthetic or natural plant sources, are needed.
Depending upon the intended oil use, various different oil
25   compositions are desired. For example, edible oil sources
containing the minimum possible amounts of saturated fatty
acids are desired for dietary reasons and alternatives to
current sources of highly saturated oil products, such as
tropical oils, are also needed.
30   One means postulated to obtain such oils and/or
modified fatty acid compositions is through the genetic
engineering of plants. However, in order to genetically
engineer plants one must have in place the means to
transfer genetic material to the plant in a stable and
35   heritable manner. Additionally, one must have nucleic acid
sequences capable of producing the desired phenotypic
result, regulatory regions capable of directing the correct
application of such sequences, and the like. Moreover, it

should be appreciated that to produce a desired modified
oils phenotype requires that the Fatty Acid Synthetase
(FAS) pathway of the plant is modified to the extent that
the ratios of reactants are modulated or changed.

5   Higher plants appear to synthesize fatty acids via a
common metabolic pathway in plant plastid organelles (i.e.,
chloroplasts, proplastids, or other related organelles) as
part of the FAS complex. Outside of plastid organelles,
fatty acids are incorporated into triglycerides and used in
10   plant membranes and in neutral lipids. In developing
seeds, where oils are produced and stored as sources of
energy for future use, FAS occurs in proplastids.

The production of fatty acids begins in the plastid
with the reaction between Acyl Carrier Protein (ACP) and
15   acetyl-CoA to produce acetyl-ACP. Through a sequence of
cylical reactions, the acetyl-ACP is elongated to 16- and
18- carbon fatty acids. The longest chain fatty acids
produced by the FAS are 18 carbons long. Monunsaturated
fatty acids are also produced in the plastid through the
20   action of a desaturase enzyme.

Common plant fatty acids, such as oleic, linoleic and
α-linolenic acids, are the result of sequential
desaturation of stearate. The first desaturation step is
the desaturation of stearoyl-ACP (C18:0) to form oleoyl-ACP
25   (C18:1) in a reaction often catalyzed by a Δ-9 desaturase,
also often referred to as a "stearoyl-ACP desaturase"
because of its high activity toward stearate the 18 carbon
acyl-ACP. The desaturase enzyme functions to add a double
bond at the ninth carbon in accordance with the following
30   reaction (I):

$$\text{Stearoyl-ACP} + \text{ferredoxin(II)} + O_2 + 2H^+ \rightarrow$$
$$\text{oleoyl-ACP} + \text{ferredoxin (III)} + 2H_2O.$$
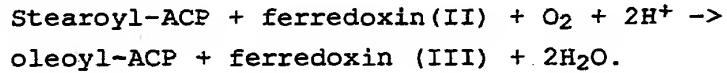
Δ-9 desaturases have been studied in partially
purified preparations from numerous plant species. Reports
35   indicate that the protein is a dimer, perhaps a homodimer,
displaying a molecular weight of 68 kD (±8 kD) by gel-
filtration and a molecular weight of 36 kD by SDS-
polyacrylamide gel electrophoresis.

In subsequent sequential steps for triglyceride
production, polyunsaturated fatty acids may be produced.
These desaturations occur outside of the plastid as a
result of the action of membrane-bound enzymes. Additional

5   double bonds are added at the twelve position carbon and
thereafter, if added, at the 15 position carbon through the
action of Δ-12 desaturase and Δ-15 desaturase,
respectively.

Obtaining nucleic acid sequences capable of producing

10   a phenotypic result in FAS, desaturation and/or
incorporation of fatty acids into a glycerol backbone to
produce an oil is subject to various obstacles including
but not limited to the identification of metabolic factors
of interest, choice and characterization of a protein

15   source with useful kinetic properties, purification of the
protein of interest to a level which will allow for its
amino acid sequencing, utilizing amino acid sequence data
to obtain a nucleic acid sequence capable of use as a probe
to retrieve the desired DNA sequence, and the preparation

20   of constructs, transformation and analysis of the resulting
plants.

Thus, the identification of enzyme targets and useful
plant sources for nucleic acid sequences of such enzyme
targets capable of modifying fatty acid compositions are

25   needed. Ideally, an enzyme target will be amenable to one
or more applications alone or in combination with other
nucleic acid sequences relating to increased/decreased oil
production, the ratio of saturated to unsaturated fatty
acids in the fatty acid pool, and/or to novel oils

30   compositions as a result of the modifications to the fatty
acid pool. Once enzyme target(s) are identified and
qualified, quantities of protein and purification protocols
are needed for sequencing. Ultimately, useful nucleic acid
constructs having the necessary elements to provide a

35   phenotypic modification and plants containing such
constructs are needed.

Relevant Literature

A 200-fold purification of *Carthamus tinctorius*
("safflower") stearoyl-ACP desaturase was reported by
McKeon & Stumpf in 1982, following the first publication of
5   their protocol in 1981. McKeon, T. & Stumpf, P.
J.Biol.Chem. (1982) *257*:12141-12147; McKeon, T. & Stumpf,
P. *Methods in Enzymol.* (1981) *71*:275-281.

BRIEF DESCRIPTION OF THE DRAWINGS

10   Fig. 1 provides amino acid sequence of fragments
relating to *C. tinctorius* desaturase. Fragments F1 through
F11 are also provided in the sequence listing as SEQ ID NO:
1 through SEQ ID NO: 11, respectively. Each fragment
represents a synthesis of sequence information from
15   peptides originating from different digests which have been
matched and aligned. In positions where there are two
amino acids indicated, the top one corresponds to that
found in the translation of the cDNA; the lower one was
detected either as a second signal at the same position of
20   one of the sequenced peptides, or as a single unambiguous
signal found in one or more of the overlapping peptides
comprising the fragment. Residues in F9 shown in lower
case letters represent positions where the called sequence
does not agree with that predicted from the cDNA, but where
25   the amino acid assignment is tentative because of the
presence of a contaminating peptide. The standard one
letter code for amino acid residues has been used. X
represents a position where no signal was detectable, and
which could be a modified residue. F1 corresponds to the
30   N-terminal sequence of the mature protein. The underlined
region in F2 is the sequence used in designing PCR primers
for probe synthesis.

Fig. 2 provides a cDNA sequence (SEQ ID NO: 12) and
the corresponding translational peptide sequence (SEQ ID
35   NO: 13) derived from *C. tinctorius* desaturase. The cDNA
sequence includes both the plastid transit peptide encoding
sequence and the sequence encoding the mature protein.

Fig. 3 provides cDNA sequence of *Ricinus communis* desaturase. Fig. 3A provides preliminary partial cDNA sequence of a 1.7 kb clone of *R. communis* desaturase (SEQ ID NO: 14). The sequence is from the 5' end of the clone.

5   Fig. 3B provides the complete cDNA sequence of the approximately 1.7 kb clone (SEQ ID NO: 15) and the corresponding translational peptide sequence (SEQ ID NO: 16).

Fig. 4 provides sequence of *Brassica campestris*

10   desaturase. Fig. 4A represents partial DNA sequence of a 1.6 kb clone pCGN3235 (SEQ ID NO: 17), from the 5' end of the clone. Fig. 4B represents partial DNA sequence of a 1.2 kb clone, pCGN3236, from the 5' end of the clone (SEQ ID NO: 18). Initial sequence for the 3' ends of the two *B.*

15   *campestris* desaturase clones indicates that pCGN3236 is a shorter cDNA for the same clone as pCGN3235. Fig. 4C provides complete cDNA sequence of *B. campestris* desaturase above, pCGN3235 (SEQ ID NO: 19) and the corresponding translational peptide sequence (SEQ ID NO: 20).

20   Fig. 5 provides preliminary partial cDNA sequence of *Simmondsia chinensis* desaturase (SEQ ID NO: 43). The translated amino acid sequence is also shown.

Fig. 6 shows the design of forward and reverse primers (SEQ ID NO: 21 through SEQ ID NO: 26) used in polymerase

25   chain reaction (PCR) from the sequence of *C. tinctorius* desaturase peptide "Fragment F2" (SEQ ID NO: 2).

Fig. 7 provides maps of desaturase cDNA clones showing selected restriction enzyme sites. Fig. 7A represents a *C. tinctorius* clone, Fig. 7B represents a *R. communis* clone,

30   and Fig. 7C represents a *B. campestris* clone.

Fig. 8 provides approximately 3.4 kb of genomic sequence of Bce4 (SEQ ID NO: 27).

Fig. 9 provides approximately 4 kb of genomic sequence of Bcg 4-4 ACP sequence (SEQ ID NO: 28).

35   Fig. 10 provides a restriction map of cloned λCGN 1-2 showing the entire napin coding region sequence as well as extensive 5' upstream and 3' downstream sequences (SEQ ID NO: 29).

SUMMARY OF THE INVENTION

By this invention, compositions and methods of use of
plant desaturase enzymes, especially Δ-9 desaturases, are
provided. Of special interest are methods and compositions
of amino acids and nucleic acid sequences related to
biologically active plant desaturases as well as sequences,
especially nucleic acid sequences, which are to be used as
probes, vectors for transformation or cloning
intermediates. Biologically active sequences may be found
in a sense or anti-sense orientation as to transcriptional
regulatory regions found in various constructs.

A first aspect of this invention relates to C.
tinctorius Δ-9 desaturase substantially free of seed
storage protein. Amino acid sequence of this desaturase is
provided in Fig. 2 and as SEQ ID NO: 13.

DNA sequence of C. tinctorius desaturase gene (SEQ ID
NO: 12) is provided, as well as DNA sequences of desaturase
genes from a Ricinus (SEQ ID NO: 14 and SEQ ID NO: 15) a
Brassica (SEQ ID NO: 17 through SEQ ID NO: 19) and a
Simmondsia (SEQ ID NO: 43) plant.

In yet a different embodiment of this invention, plant
desaturase cDNA of at least 10 nucleotides or preferably at
least 20 nucleotides and more preferably still at least 50
nucleotides, known or homologously related to known Δ-9
desaturase(s) is also provided. The cDNA encoding
precursor desaturase or, alternatively, biologically
active, mature desaturase is provided herein.

Methods to use nucleic acid sequences to obtain other
plant desaturases are also provided. Thus, a plant
desaturase may be obtained by the steps of contacting a
nucleic acid sequence probe comprising nucleotides of a
known desaturase sequence and recovery of DNA sequences
encoding plant desaturase having hybridized with the probe.

This invention also relates to methods for obtaining
plant Δ-9 desaturase by contacting an antibody specific to
a known desaturase, such as C. tinctorius stearoyl-ACP

desaturase, with a candidate plant stearoyl-ACP desaturase under conditions conducive to the formation of an antigen:antibody immunocomplex and the recovery of the candidate plant stearoyl-ACP desaturase which reacts

5   thereto.

In a further aspect of this invention DNA constructs comprising a first DNA sequence encoding a plant desaturase and a second DNA sequence which is not naturally found joined to said plant desaturase are provided. This

10   invention also relates to the presence of such constructs in host cells, especially plant host cells. In yet a different aspect, this invention relates to transgenic host cells which have an expressed desaturase therein.

Constructs of this invention may contain, in the 5' to

15   3' direction of transcription, a transcription initiation control regulatory region capable of promoting transcription in a host cell and a DNA sequence encoding plant desaturase. Transcription initiation control regulatory regions capable of expression in prokatyotic or

20   eukaryotic host cells are provided. Most preferred are transcription initiation control regions capable of expression in plant cells, and more preferred are transcription and translation initiation regions preferentially expressed in plant cells during the period

25   of lipid accumulation. The DNA sequence encoding plant desaturase of this invention may be found in either the sense or anti-sense orientation to the transcription initiation control region.

Specific constructs, expression cassettes having in

30   the 5' to 3' direction of transcription, a transcription and translation initiation control regulatory region comprising sequence immediately 5' to a structural gene preferentially expressed in plant seed during lipid accumulation, a DNA sequence encoding desaturase, and

35   sequence 3' to the structural gene are also provided. The construct may preferably contain DNA sequences encoding plant desaturase obtainable (included obtained) from *Carthamus*, *Rininus*, *Brassica* or *Simmondsia* Δ-9 desaturase

genes. Transcription and translation initiation control
regulatory regions are preferentially obtained from
structural genes preferentially expressed in plant embryo
tissue such as napin, seed-ACP or Bce-4.

5   By this invention, methods and constructs to inhibit
the production of endogenous desaturase are also provided.
For example, an anti-sense construct comprising, in the 5'
to 3' direction of transcription, a transcription
initiation control regulatory region functional in a plant

10   cell, and an anti-sense DNA sequence encoding a portion of
a plant Δ-9 desaturase may be integrated into a plant host
cell to decrease desaturase levels.

In yet a different embodiment, this invention is
directed to a method of producing plant desaturase in a

15   host cell comprising the steps of growing a host cell
comprising an expression cassette, which would contain in
the direction of transcription, a) a transcription and
translation initiation region functional in said host cell,
b) the DNA sequence encoding a plant desaturase in reading

20   frame with said initiation region, and c) and a transcript
termination region functional in said host cell, under
conditions which will promote the expression of the plant
desaturase. Cells containing a plant desaturase as a
result of the production of the plant desaturase encoding

25   sequence and also contemplated herein.

By this invention, a method of modifying fatty acid
composition in a host plant cell from a given level of
fatty acid saturation to a different level of fatty acid
saturation is provided by growing a host plant cell having

30   integrated into its genome a recombinant DNA sequence
encoding a plant desaturase in either a sense or anti-sense
orientation under control of regulatory elements functional
in said plant cell during lipid accumulation under
conditions which will promote the activity of said

35   regulatory elements. Plant cells having such a modified
level of fatty acid saturation are also contemplated
hereunder. Oilseeds having such a modified level of fatty

acid saturation and oils produced from such oilseeds are
further provided.

DETAILED DESCRIPTION OF THE INVENTION

5   A plant desaturase of this invention includes any
sequence of amino acids, such as a protein, polypeptide, or
peptide fragment, obtainable from a plant source which is
capable of catalyzing the insertion of a first double bond
into a fatty acyl-ACP moiety in a plant host cell, i.e., *in*
10   *vivo*, or in a plant cell-like environment, i.e. *in vitro*.
"A plant cell-like environment" means that any necessary
conditions are available in an environment (i.e., such
factors as temperatures, pH, lack of inhibiting substances)
which will permit the enzyme to function   In particular,
15   this invention relates to enzymes which add such a first
double bond at the ninth carbon position in a fatty acyl-
ACP chain.   There may be similar plant desaturase enzymes
of this invention with different specificities, such as the
Δ-12 desaturase of carrot.
20   Nucleotide sequences encoding desaturases may be
obtained from natural sources or be partially or wholly
artificially synthesized.   They may directly correspond to
a desaturase endogenous to a natural plant source or
contain modified amino acid sequences, such as sequences
25   which have been mutated, truncated, increased or the like.
Desaturases may be obtained by a variety of methods,
including but not limited to, partial or homogenous
purification of plant extracts, protein modeling, nucleic
acid probes, antibody preparations and sequence
30   comparisons.   Typically a plant desaturase will be derived
in whole or in part from a natural plant source.
Of special interest are Δ-9 desaturases which are
obtainable, including those with are obtained, from
Cartharmus, Ricinus, Simmondsia, or *Brassica,* for example
35   *C. tinctorius, R. communis, S. chinensis* and *B. campestris,*
respectively, or from plant desaturases which are
obtainable through the use of these sequences.
"Obtainable" refers to those desaturases which have

sufficiently similar sequences to that of the native
sequences provided herein to provide a biologically active
desaturase.

      Once a DNA sequence which encodes a desaturase is
5   obtained, it may be employed as a gene of interest in a
nucleic acid construct or in probes in accordance with this
invention. A desaturase may be produced in host cells for
harvest or as a means of effecting a contact between the
desaturase and its substrate. Constructs may be designed
10 to produce desaturase in either prokaryotic or eukaryotic
cells. Plant cells containing recombinant constructs
encoding biologically active desaturase sequences, both
expression and anti-sense constructs, as well as plants and
cells containing modified levels of desaturase proteins are
15 of special interest.   For use in a plant cell, constructs
may be designed which will effect an increase or a decrease
in amount of endogenous desaturase available to a plant
cell transformed with such a construct.

      Where the target gene encodes an enzyme, such as a
20 plant desaturase, which is already present in the host
plant, there are inherent difficulties in analyzing mRNA,
engineered protein or enzyme activity, and modified fatty
acid composition or oil content in plant cells, especially
in developing seeds; each of which can be evidence of
25 biological activity. This is because the levels of the
message, enzyme and various fatty acid species are changing
rapidly during the stage where measurements are often made,
and thus it can be difficult to discriminate between
changes brought about by the presence of the foreign gene
30 and those brought about by natural developmental changes in
the seed. Where an expressed Δ-9 desaturase DNA sequence
is derived from a plant species heterologous to the plant
host into which the sequence is introduced and has a
distinguishable DNA sequence, it is often possible to
35 specifically probe for expression of the foreign gene with
oligonucleotides complimentary to unique sequences of the
inserted DNA/RNA. And, if the foreign gene codes for a
protein with slightly different protein sequence, it may be

possible to obtain antibodies which recognize unique
epitopes on the engineered protein. Such antibodies can be
obtained by mixing the antiserum to the foreign protein
with extract from the host plant, or with extracts
5   containing the host plant enzyme. For example, one can
isolate antibodies uniquely specific to a *C. tinctorius* Δ-
9 desaturase by mixing antiserum to the desaturase with an
extract containing a *Brassica* Δ-9 desaturase. Such an
approach will allow the detection of *C. tinctorius*
10   desaturase in *Brassica* plants transformed with the *C.
tinctorius* desaturase gene. In plants expressing an
endogenous gene in an antisense orientation, the problem is
slightly different. In this case, there are no specific
reagents to measure expression of a foreign protein.
15   However, one is attempting to measure a decrease in an
enzyme activity that normally is increasing during
development. This makes detection of expression a simpler
matter. In the final seed maturation phase, enzyme
activities encoded by genes affecting oil composition
20   usually disappear and cannot be detected in final mature
seed. Analysis of the fatty acid content may be preformed
by any manner known to those skilled in the art, including
gas chromatography, for example.
          By increasing the amount of desaturase available in
25   the plant cell, an increased percentage of unsaturated
fatty acids may be provided; by decreasing the amount of
desaturase, an increased percentage of saturated fatty
acids may be provided. (Modifications in the pool of fatty
acids available for incorporation into triglycerides may
30   likewise affect the composition of oils in the plant cell.)
Thus, an increased expression of desaturase in a plant cell
may result in increased proportion of fatty acids, such as
one or more of palmitoleate (C16:1), oleate (C18:1),
linoleate (C18:2) and linolenate (C18:3) are expected. Of
35   special interest is the production of triglycerides having
increased levels of oleate. Using anti-sense technology,
alternatively, a decrease in the amount of desaturase
available to the plant cell is expected, resulting in a

higher percentage of saturates such as one or more of
laurate (C12:0), myristate (C14:0), palmitate (C16:0),
stearate (C18:0), arachidate (C20:0), behemate (C22:0) and
lignocerate (C24:0). Of special interest is the production

5   of triglycerides having increased levels of stearate or
palmitate and stearate. In addition, the production of a
variety of ranges of such saturates is desired. Thus,
plant cells having lower and higher levels of stearate
fatty acids are contemplated. For example, fatty acid

10   compositions, including oils, having a 10% level of
stearate as well as compositions designed to have up to a
60% level of stearate or other such modified fatty acid(s)
composition are contemplated.

        The modification of fatty acid compositions may also

15   affect the fluidity of plant membranes. Different lipid
concentrations have been observed in cold-hardened plants,
for example. By this invention, one may be capable of
introducing traits which will lend to chill tolerance.
Constitutive or temperature inducible transcription

20   initiation regulatory control regions may have special
applications for such uses.

        Other applications for use of cells or plants
producing desaturase may also be found. For example,
potential herbicidal agents selective for plant desaturase

25   may be obtained through screening to ultimately provide
environmentally safe herbicide products. The plant
desaturase can also be used in conjunction with chloroplast
lysates to enhance the production and/or modify the
composition of the fatty acids prepared *in vitro*. The

30   desaturase can also be used for studying the mechanism of
fatty acid formation in plants and bacteria. For these
applications, constitutive promoters may find the best use.

        Constructs which contain elements to provide the
transcription and translation of a nucleic acid sequence of

35   interest in a host cell are "expression cassettes".
Depending upon the host, the regulatory regions will vary,
including regions from structural genes from viruses,
plasmid or chromosomal genes, or the like. For expression

in prokaryotic or eukaryotic microorganisms, particularly
unicellular hosts, a wide variety of constitutive or
regulatable promoters may be employed. Among
transcriptional initiation regions which have been
5   described are regions from bacterial and yeast hosts, such
as *E. coli, B. subtilis, Saccharomyces cerevisiae,*
including genes such as β-galactosidase, T7 polymerase, trp
E and the like.

A recombinant construct for expression of desaturase
10 in a plant cell ("expression cassette") will include, in
the 5' to 3' direction of transcription, a transcription
and translation initiation control regulatory region (the
transcriptional and translational initiation regions
together often also known as a "promoter") functional in a
15 plant cell, a nucleic acid sequence encoding a plant
desaturase, and a transcription termination region.
Numerous transcription initiation regions are available
which provide for a wide variety of constitutive or
regulatable, e.g., inducible, transcription of the
20 desaturase structural gene. Among transcriptional
initiation regions used for plants are such regions
associated with cauliflower mosaic viruses (35S, 19S), and
structural genes such as for nopaline synthase or mannopine
synthase or napin and ACP promoters, etc. The
25 transcription/translation initiation regions corresponding
to such structural genes are found immediately 5' upstream
to the respective start codons. Thus, depending upon the
intended use, different promoters may be desired.

Of special interest in this invention are the use of
30 promoters which are capable of preferentially expressing
the desaturase in seed tissue, in particular, at early
stages of seed oil formation. Examples of such seed-
specific promoters include the region immediately 5'
upstream of napin or seed ACP genes, such as described in
35 co-pending USSN 147,781, and the Bce-4 gene such as
described in co-pending USSN 494,722. Alternatively, the
use of the 5' regulatory region associated with an
endogenous plant desaturase structural gene and/or the

transcription termination regions found immediately 3'
downstream to the gene, may often be desired.

In addition, for some applications, use of more than
one promoter may be desired. For example, one may design a
5   dual promoter expression cassette each promoter having a
desaturase sequence under its regulatory control. For
example, the combination of an ACP and napin cassette could
be useful for increased production of desaturase in a seed-
specific fashion over a longer period of time than either
10 individually.

To decrease the amount of desaturase found in a plant
host cell, anti-sense constructs may be prepared and then
inserted into the plant cell. By "anti-sense" is meant a
DNA sequence in the 5' to 3' direction of transcription in
15 relation to the transcription initiation region, which
encodes a sequence complementary to the sequence of a
native desaturase. It is preferred that an anti-sense
plant desaturase sequence be complementary to a plant
desaturase gene indigenous to the plant host. Sequences
20 found in an anti-sense orientation may be found in
constructs providing for transcription or transcription and
translation of the DNA sequence encoding the desaturase,
including expression cassettes. Constructs having more
than one desaturase sequence under the control of more than
25 one promoter or transcription initiation region may also be
employed with desaturase constructs. Various transcription
initiation regions may be employed. One of ordinary skill
in the art can readily determine suitable regulatory
regions. Care may be necessary in selecting transcription
30 initiation regions to avoid decreasing desaturase activity
in plant cells other than oilseed tissues. Any
transcription initiation region capable of directing
expression in a plant host which causes initiation of
adequate levels of transcription selectively in storage
35 tissues during seed development for example, should be
sufficient. As such, seed specific promoters may be
desired. Other manners of decreasing the amount of
endogenous plant desaturase, such as ribozymes or the

screening of plant cells transformed with constructs for
rare events containing sense sequences which in fact act to
decrease desaturase expression, are also contemplated
herein. Other analogous methods may be applied by those of
5   ordinary skill in the art.

By careful selection of plants, transformants having
particular oils profiles may be obtained. This may in part
depend upon the qualities of the transcription initiation
region(s) employed or may be a result of culling
10 transformation events to exploit the variabilities of
expression observed.

In order to obtain the nucleic acid sequences encoding
C. tinctorius desaturase, a protein preparation free of a
major albumin-type contaminant is required. As
15 demonstrated more fully in the Examples, the protocols of
McKeon and Stumpf, supra, result in a preparation
contaminated with a seed storage protein. Removal of the
protein contaminant may be effected by application of a
reverse-phase HPLC, or alternatively, by application of a
20 reduction and alkylation step followed by electrophoresis
and blotting, for example. Other purification methods may
be employed as well, now that the presence of the
contaminant is confirmed and various properties thereof
described. Once the purified desaturase is obtained it may
25 be used to obtain the corresponding amino acid and/or
nucleic acid sequences thereto in accordance with methods
familiar to those skilled in the art. Approximately 90% of
the total amino acid sequence of the C. tinctorius
desaturase is provided in Fig. 1 and in SEQ ID NOS: 1-11.
30 The desaturase produced in accordance with the subject
invention can be used in preparing antibodies for assays
for detecting plant desaturase from other sources.

A nucleic acid sequence of this invention may include
genomic or cDNA sequence and mRNA. A cDNA sequence may or
35 may not contain pre-processing sequences, such as transit
peptide sequences. Transit peptide sequences facilitate
the delivery of the protein to a given organelle and are

cleaved from the amino acid moiety upon entry into the
organelle, releasing the "mature" sequence.

In Fig. 2 and SEQ ID NO: 13, the sequence of the *C.
tinctorius* desaturase precursor protein is provided; both

5   the transit peptide and mature protein sequence are shown.
Also provided in this invention are cDNA sequences relating
to *R. communis* desaturase (Fig.3 and SEQ ID NOS: 14-15), *B.
campestris* desaturase (Fig. 4 and SEQ ID NOS: 17-19) and *S.
chinesis* (Fig. 5 and SEQ ID NOS: 43).

10   The use of the precursor cDNA sequence is preferred in
desaturase expression cassettes. In addition, desaturase
transit peptide sequences may be employed to translocate
other proteins of interest to plastid organelles for a
variety of uses, including the modulation of other enzymes

15   related to the FAS pathway. See, European Patent
Application Publication No. 189,707.

As described in more detail below, the complete
genomic sequence of a desaturase may be obtained by the
screening of a genomic library with a desaturase cDNA probe

20   and isolating those sequences which regulate expression in
seed tissue. In this manner, the transcription,
translation initiation regions and/or transcript
termination regions of the desaturase may be obtained for
use in a variety of DNA constructs, with or without the

25   respective desaturase structural gene.

Other nucleic acid sequences "homologous" or "related"
to DNA sequences encoding other desaturases are also
provided. "Homologous" or "related" includes those nucleic
acid sequences which are identical or conservatively

30   substituted as compared to the exemplified *C. tinctorius,
R. communis, S. chinesis* or *B. campestris* desaturase
sequences of this invention or a plant desaturase which has
in turn been obtained from a plant desaturase of this
invention. By conservatively substituted is meant that

35   codon substitutions encode the same amino acid, as a result
of the degeneracy of the DNA code, or that a different
amino acid having similar properties to the original amino
acid is substituted. One skilled in the art will readily

recognize that antibody preparations, nucleic acid probes
(DNA and RNA) sequences encoding and the like may be
prepared and used to screen and recover desaturase from
other plant sources. Typically, nucleic acid probes are
5   labeled to allow detection, preferably with radioactivity
although enzymes or other methods may also be used. For
immunological screening methods, antibody preparations
either monoclonal or polyclonal are utilized. Polyclonal
antibodies, although less specific, typically are more
10   useful in gene isolation. For detection, the antibody is
labeled using radioactivity or any one of a variety of
second antibody/enzyme conjugate systems that are
commercially available. Examples of some of the available
antibody detection systems are described by Oberfilder
15   (*Focus* (1989) BRL Life Technologies, Inc., *11*:1-5).

A "homologous" or "related" nucleic acid sequence will
show at least about 60% homology, and more preferably at
least about 70% homology, between the known desaturase
sequence and the desired candidate plant desaturase of
20   interest, excluding any deletions which may be present.
Homology is determined upon comparison of sequence
information, nucleic acid or amino acid, or through
hybridization reactions. Amino acid sequences are
considered homologous by as little as 25% sequence identity
25   between the two complete mature proteins. (*See generally*,
Doolittle, R.F., of URFS and ORFS, University Science
Books, CA, 1986.)

Oligonucleotide probes can be considerably shorter
than the entire sequence, but should be at least about 10,
30   preferably at least about 15, more preferably at least 20
nucleotides in length. When shorter length regions are
used for comparison, a higher degree of sequence identity
is required than for longer sequences. Shorter probes are
often particularly useful for polymerase chain reactions
35   (PCR), especially when highly conserved sequences can be
identified. (*See*, Gould, et *al*., *PNAS USA* (1989) *86*:1934-
1938.) Longer oligonucleotides are also useful, up to the
full length of the gene encoding the polypeptide of

interest. When longer nucleic acid fragments are employed
(>100 bp) as probes, especially when using complete or
large cDNA sequences, one would screen with low
stringencies (for example 40-50°C below the melting
5   temperature of the probe) in order to obtain signal from
the target sample with 20-50% deviation, i.e., homologous
sequences. (See, Beltz, et al., *Methods in Enzymology*
(1983) *100*:266-285.) Both DNA and RNA probes can be used.
        A genomic library prepared from the plant source of
10   interest may be probed with conserved sequences from a
known desaturase to identify homologously related
sequences. Use of the entire cDNA may be employed if
shorter probe sequences are not identified. Positive
clones are then analyzed by restriction enzyme digestion
15   and/or sequencing. When a genomic library is used, one or
more sequences may be identified providing both the coding
region, as well as the transcriptional regulatory elements
of the desaturase gene from such plant source. In this
general manner, one or more sequences may be identified
20   providing both the coding region, as well as the
transcriptional regulatory elements of the desaturase gene
from such plant source.
        In use, probes are typically labeled in a detectable
manner (for example with ^{32}P-labeled or biotinylated
25   nucleotides) and are incubated with single-stranded DNA or
RNA from the plant source in which the gene is sought,
although unlabeled oligonucleotides are also useful.
Hybridization is detected by means of the label after
single-stranded and double-stranded (hybridized) DNA or
30   DNA/RNA have been separated, typically using nitrocellulose
paper or nylon membranes. Hybridization techniques suitable
for use with oligonucleotides are well known to those
skilled in the art. Thus, plant desaturase genes may be
isolated by various techniques from any convenient plant.
35   Plant desaturase of developing seed obtained from other
oilseed plants, such as soybean, coconut, oilseed rape,
sunflower, oil palm, peanut, cocoa, cotton, corn and the
like are desired as well as from non-traditional oil

sources, including but not limited to spinach chloroplast, avocado mesocarp, cuphea, California Bay, cucumber, carrot, meadowfoam, *Oenothera* and *Euglena gracillis*.

Once the desired plant desaturase sequence is obtained, it may be manipulated in a variety of ways. Where the sequence involves non-coding flanking regions, the flanking regions may be subjected to resection, mutagenesis, etc. Thus, transitions, transversions, deletions, and insertions may be performed on the naturally occurring sequence. In addition, all or part of the sequence may be synthesized, where one or more codons may be modified to provide for a modified amino acid sequence, or one or more codon mutations may be introduced to provide for a convenient restriction site or other purpose involved with construction or expression. The structural gene may be further modified by employing synthetic adapters, linkers to introduce one or more convenient restriction sites, or the like.

Recombinant constructs containing a nucleic acid sequence encoding a desaturase of this invention may be combined with other, i.e. "heterologous," DNA sequences in a variety of ways. By heterologous DNA sequences is meant any DNA sequence which is not naturally found joined to the native desaturase, including combinations of DNA sequences from the same plant of the plant desaturase which are not naturally found joined together. In a preferred embodiment, the DNA sequence encoding a plant desaturase is combined in a DNA construct having, in the 5' to 3' direction of transcription, a transcription initiation control region capable of promoting transcription in a host cell, and a DNA sequence encoding a desaturase in either a sense or anti-sense orientation. As described in more detail elsewhere, a variety of regulatory control regions containing transcriptional or transcriptional and translational regions may be employed, including all or part of the non-coding regions of the plant desaturase.

The open reading frame coding for the plant desaturase or functional fragment thereof will be joined at its 5' end

to a transcription initiation regulatory control region.
In some instances, such as modulation of plant desaturase
via a desaturase in an anti-sense orientation, a
transcription initiation region or transcription/
5   translation initiation region may be used. In embodiments
wherein the expression of the desaturase protein is desired
in a plant host, a transcription/ translation initiation
regulatory region, is needed. Additionally, modified
promoters, i.e., having transcription initiation regions
10   derived from one gene source and translation initiation
regions derived from a different gene source or enhanced
promoters, such as double 35S CaMV promoters, may be
employed for some applications.

     As described above, of particular interest are those
15   5' upstream non-coding regions which are obtained from
genes regulated during seed maturation, particularly those
preferentially expressed in plant embryo tissue, such as
ACP-and napin-derived transcription initiation control
regions. Such regulatory regions are active during lipid
20   accumulation and therefore offer potential for greater
control and/or effectiveness to modify the production of
plant desaturase and/or modification of the fatty acid
composition.   Especially of interest are transcription
initiation regions which are preferentially expressed in
25   seed tissue, i.e., which are undetectable in other plant
parts. For this purpose, the transcript initiation region
of acyl carrier protein isolated from B. campestris seed
and designated as "Bcg 4-4" and an unidentified gene
isolated from B. campestris seed and designated as "Bce-4"
30   are also of substantial interest.

     Briefly, Bce4 is found in immature embryo tissue at
least as early as 11 days after anthesis (flowering),
peaking about 6 to 8 days later or 17-19 days post-
anthesis, and becoming undetectable by 35 days post-
35   anthesis. The timing of expression of the Bce4 gene
closely follows that of lipid accumulation in seed tissue.
Bce4 is primarily detected in seed embryo tissue and to a
lesser extent found in the seed coat. Bce4 has not been

detected in other plant tissues tested, root, stem and
leaves.

　　　Approximately 3.4 kb genomic sequence of Bce4 is
provided in Fig. 8 and as SEQ ID NO: 27, including about 1
5   kb 5' to the structural gene, about 0.3 kb of the Bce4
coding gene sequence, and about 2.1 kb of the non-coding
regulatory 3' sequence. Bce4 transcript initiation regions
will contain at least 1 kb and more preferably about 5 to
about 7.5 kb of sequence immediately 5' to the Bce4
10   structural gene.

　　　The Bcg 4-4 ACP message presents a similar expression
profile to that of Bce4 and, therefore, also corresponds to
lipid accumulation in the seed tissue. Bcg 4-4 is not
found in the seed coat and may show some differences in
15   expression level, as compared to Bce4, when the Bcg 4-4 5'
non-coding sequence is used to regulate transcription or
transcription and translation of a plant stearoyl-ACP
desaturase of this invention. Genomic sequence of Bcg 4-4
is provided in Fig. 9 and as SEQ ID NO: 28, including about
20   1.5 kb 5' to the structural gene, about   1.2 kb of the
Bcg 4-4 (ACP) structural gene sequence, and about 1.3 kb of
the non-coding regulatory 3' sequence.

　　　The napin 1-2 message is found in early seed
development and thus, also offers regulatory regions which
25   can offer preferential transcriptional regulation of a
desired DNA sequence of interest such as the plant
desaturase DNA sequence of this invention during lipid
accumulation. Napins are one of the two classes of storage
proteins synthesized in developing *Brassica* embryos
30   (Bhatty, et *al., Can J. Biochem.* (1968) *46*:1191-1197) and
have been used to direct tissue-specific expression when
reintroduced into the *Brassica* genome (Radke, et *al.,*
Theor. Appl. Genet. (1988) 75:685-694). Genomic sequence
of napin 1-2 is provided in Fig. 10 and as SEQ ID NO: 29,
35   including about 1.7 kb 5' to the structural gene and about
1.3 kb of the non-coding regulatory 3' sequence

　　　Regulatory transcript termination regions may be
provided in DNA constructs of this invention as well.

Transcript termination regions may be provided by the DNA
sequence encoding the plant desaturase or a convenient
transcription termination region derived from a different
gene source, especially the transcript termination region
5 which is naturally associated with the transcript
initiation region. The transcript termination region will
contain at least about 1 kb, preferably about 3 kb of
sequence 3' to the structural gene from which the
termination region is derived.

10   In developing the DNA construct, the various
components of the construct or fragments thereof will
normally be inserted into a convenient cloning vector which
is capable of replication in a bacterial host, e.g., *E.
coli*. Numerous vectors exist that have been described in
15 the literature. After each cloning, the plasmid may be
isolated and subjected to further manipulation, such as
restriction, insertion of new fragments, ligation,
deletion, insertion, resection, etc., so as to tailor the
components of the desired sequence. Once the construct has
20 been completed, it may then be transferred to an
appropriate vector for further manipulation in accordance
with the manner of transformation of the host cell.

     Normally, included with the DNA construct will be a
structural gene having the necessary regulatory regions for
25 expression in a host and providing for selection of
transformant cells. The gene may provide for resistance to
a cytotoxic agent, e.g. antibiotic, heavy metal, toxin,
etc., complementation providing prototrophy to an
auxotrophic host, viral immunity or the like. Depending
30 upon the number of different host species into which the
expression construct or components thereof are introduced,
one or more markers may be employed, where different
conditions for selection are used for the different hosts.

     The manner in which the DNA construct is introduced
35 into the plant host is not critical to this invention. Any
method which provides for efficient transformation may be
employed. Various methods for plant cell transformation
include the use of Ti- or Ri-plasmids, microinjection,

electroporation, liposome fusion, DNA bombardment or the
like. In many instances, it will be desirable to have the
construct bordered on one or both sides by T-DNA,
particularly having the left and right borders, more
5 particularly the right border. This is particularly useful
when the construct uses *A. tumefaciens* or *A. rhizogenes* as
a mode for transformation, although the T-DNA borders may
find use with other modes of transformation.

Where *Agrobacterium* is used for plant cell
10 transformation, a vector may be used which may be
introduced into the *Agrobacterium* host for homologous
recombination with T-DNA or the Ti- or Ri-plasmid present
in the *Agrobacterium* host. The Ti- or Ri-plasmid
containing the T-DNA for recombination may be armed
15 (capable of causing gall formation) or disarmed (incapable
of causing gall formation), the latter being permissible,
so long as the *vir* genes are present in the transformed
Agrobacterium host. The armed plasmid can give a mixture
of normal plant cell and gall.

20 A preferred method for the use of *Agrobacterium* as the
vehicle for transformation of plant cells employs a vector
having a broad host range replication system, at least one
T-DNA boundary and the DNA sequence or sequences of
interest. Commonly used vectors include pRK2 or
25 derivatives thereof. See, for example, Ditta et *al.*, *PNAS
USA*, (1980) 77:7347-7351 and EPA 0 120 515, which are
incorporated herein by reference. Normally, the vector
will be free of genes coding for opines, oncogenes and *vir-*
genes. Included with the expression construct and the T-
30 DNA will be one or more markers, which allow for selection
of transformed *Agrobacterium* and transformed plant cells.
A number of markers have been developed for use with plant
cells, such as resistance to chloramphenicol, the
aminoglycoside G418, hygromycin, or the like. The
35 particular marker employed is not essential to this
invention, one or another marker being preferred depending
on the particular host and the manner of construction.

The expression constructs may be employed with a wide variety of plant life, particularly plant life involved in the production of vegetable oils. These plants include, but are not limited to rapeseed, sunflower, *C. tinctorius*,
5   cotton, *Cuphea*, peanut, soybean, oil palm and corn. Anti-sense constructs may be employed in such plants which share complementarity between the endogenous sequence and the anti-sense desaturase. Of special interest is the use of an anti-sense construct having a *B. campestris* desaturase
10 in rapeseed, including *B. campestris* and *B. napus*.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an
15 appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be grown to seed and the seed
20 used to establish repetitive generations and for isolation of vegetable oils compositions. A variety of stable genetic lines having fixed levels of saturation may be obtained and integrated into a traditional breeding program. Hemizygous and heterozygous lines or homozygous
25 lines may demonstrate different useful properties for oil production and/or breeding. For example, saturation levels may be increased up to 2-fold by the development of homozygous plants as compared with heterozygous (including hemizygous) plants.

30 The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

35   **EXAMPLES**

MATERIALS

Commercially available biological chemicals and chromatographic materials, including BSA, catalase (twice

25

crystalized from bovine liver), spinach ferredoxin,
ferredoxin-NADP$^+$ oxidoreductase (spinach leaf), NADPH,
unlabeled fatty acids, DEAE-cellulose (Whatman DE-52) CNBr-
activated Sepharose 4B, and octyl-Sepharose, and Reactive

5   Blue Agarose are from Sigma (St. Louis, MO).
Triethylamine, trichloroacetic acid, guanidine-HCl, and
hydrazine-hydrate are also from Sigma. Proteolytic
enzymes, including endoproteinases lysC, gluC, and aspN are
sequencing grade enzymes obtained from Boehringer Mannheim

10   (Indianapolis, IN). Organic solvents, including acetone,
acetonitrile, methanol, ether and petroleum ether are
purchased from J.T. Baker (Phillipsburg, NJ); concentrated
acids and sodium sulfate are also from J.T. Baker
(Phillipsburg, NJ). HPLC grade acetonitrile and

15   trifluoracetic acid (TFA) are obtained from Burdick and
Jackson (Muskegon, MI), and from Applied Biosystems (Foster
City, CA), respectively. Radiochemicals, including
[9,10(n)-^{3}H] oleic acid (10mCi/μmol) and [^{3}H]-iodoacetic
acid (64Ci/mol) are from New England Nuclear (Boston, MA).

20   Phenacyl-8 Reagent (bromoacetophenone with a crown ether
catalyst) used to prepare phenacyl esters of the fatty
acids for analysis are from Pierce (Rockford, IL). C18
reversed-phase thin-layer chromatography plates are from
Whatman (Clifton, NJ).

25   Acyl carrier protein (ACP) and acyl-ACP synthase are
isolated from *E. coli* strain K-12 as described by Rock and
Cronan (Rock and Cronan, *Methods in Enzymol* (1981) 71:341-
351 and Rock et al., *Methods in Enzymol.* (1981) 72:397-
403). The *E. coli* is obtainable from Grain Processing

30   (Iowa) as frozen late-logarithmic phase cells.
       [9,10(n)-^{3}H]stearic acid is synthesized by reduction
of [9,10(n)-^{3}H]oleic acid with hydrazine hydrate
essentially as described by Johnson and Gurr (*Lipids* (1971)
6:78-84). [9,10(n)-^{3}H]oleic acid (2 mCi), supplemented

35   with 5.58mg unlabeled oleic acid to give a final specific
radioactivity of 100mCi/mmol, is dissolved in 2ml of
acetonitrile, acidified with 40μl of glacial acetic acid,
and heated to 55°C. Reduction is initiated with 100μl of

26

60% (w/w) hydrazine hydrate; oxygen is bubbled through the
mixture continuously. After each hour acetonitrile is
added to bring the volume back to 2ml and an additional
100μl of hydrazine hydrate is added. At the end of 5 hr.
5   the reaction is stopped by addition of 3ml of 2M HCl. The
reaction products are extracted with three 3ml aliquots of
petroleum ether and the combined ether extracts are washed
with water, dried over sodium sulfate and evaporated to
dryness. The dried reaction products are redissolved in
10 1.0ml acetonitrile and stored at -20°C. The distribution
of fatty acid products in a 15μl aliquot is determined by
preparation of phenacyl esters, which are then analyzed by
thin layer chromatography on C-18 reverse phase plates
developed with methanol:water:95:5 (v/v). Usually
15 reduction to [9,10(n)-^{3}H]stearic acid is greater than 90%,
a small amount of unreacted oleic acid may remain. The
analysis is used to establish fraction of the total
radioactivity that is present as stearate, and thereby to
determine the exact substrate concentration in the enzyme
20 assay.

      Acyl-ACP substrates, including [9,10(n)-^{3}H] stearoyl-
ACP are prepared and purified by the enzymatic synthesis
procedure of Rock, Garwin, and Cronan (*Methods in Enzymol.*
(1981) *72*:397-403).

25   Acyl carrier protein was covalently bound to Sepharose
4B by reaction of highly purified ACP with CNBr-activated
Sepharose 4B as described by McKeon and Stumpf (*J. Biol.
Chem.* (1982) *257*:12141-12147).

30 **Example 1**

      In this example, an initial purification of *C.
tinctorius* (safflower) desaturase, following the method of
McKeon and Stumpf (*J. Biol. Chem.* (1982) *257*:12141-12142),
is described.
35   *Assay:* In each of the following steps, the presence
of the enzyme is detected radiometrically by measuring
enzyme-catalyzed release of tritium from [9,10(n)-

^{3}H]stearoyl-ACP. Preparation of this substrate is
described in "Materials" above.

The assay is performed by mixing 150μl water, 5ml
dithiothreitol (100mM, freshly prepared in water), 10μl

5   bovine serum albumin (10mg/ml in water), 15μl NADPH (25mM,
freshly prepared in 0.1M Tricine-HCl, pH 8.2), 25μl spinach
ferredoxin (2mg/ml Sigma Type III in water), 3μl
NADPH:ferredoxin oxidoreductase (2.5 units/ml from Sigma),
and 1 μl bovine liver catalase (800,000 units/ml from

10 Sigma); after 10 min at room temperature, this mixture is
added to a 13x100 mm screw-cap test tube containing 250μl
sodium 1,4-piperazinediethanesulfonate (0.1M, pH 6.0).
Finally, 10μl of the sample to be assayed is added and the
reaction is started by adding 30μl of the substrate,

15 [9,10(n)-^{3}H]stearoyl-ACP (100μCi/μmol, 10μM in 0.1M sodium
1,4-piperazinediethanesulfonate, pH 5.8). After sealing
with a cap, the reaction is allowed to proceed for 10 min.
while shaking at 23°C. The reaction is terminated by
addition of 1.2ml of 5.8% tricholoracetic acid and the

20 resulting precipitated acyl-ACP's are removed by
centrifugation. The tritium released into the aqueous
supernatant by the desaturase reaction is measured by
liquid scintillation spectrometry. One unit of activity is
defined as the amount of enzyme required to convert 1μmol

25 of stearoyl-ACP to oleoyl-ACP, or to release 4μg-atoms of
^{3}H per minute.

Source tissue: Developing *C. tinctorius* seeds from
greenhouse grown plants are harvested between 16 and 18
days after flowering, frozen in liquid nitrogen and stored

30 at -70°C until extracted.

Acetone Powder: Approximately 50g of frozen seeds
are ground in liquid nitrogen and sieved to remove large
seed coat pieces to provide a fine embryo powder. The
powder is washed with acetone on a Buchner funnel until all

35 yellow color is absent from the filtrate. The powder is
then air dried and further processed as described below, or
may be stored frozen for at least a year at -70°C without
loss of enzyme activity.

     Acetone Powder Extract: The dried acetone powder is
weighed and triturated with ten times its weight of 20mM
potassium phosphate, pH 6.8; the mixture is then
centrifuged at 12,000 x g for 20 minutes and decanted
5   through a layer of Miracloth (Calbiochem, La Jolla, CA).
     Ion Exchange Chromatography: The acetone powder
extract is then applied to a DEAE-cellulose column (Whatman
DE-52) (1.5 x 12 cm) equilibrated with 20mM potassium
phosphate, pH 6.8. The pass-through and a wash with one
10 column-volume (20ml) of buffer are pooled.
     Affinity Chromatography: An affinity matrix for
purification of the desaturase is prepared by reacting
highly purified *E. coli* ACP, with CNBr-activated Sepharose
4B (Sigma). ACP (120mg) is reduced by treatment with 1mM
15 dithiothreitol for 30 min on ice, and then desalted on
Sephadex G-10 (Pharmacia) equilibrated with 0.1M sodium
bicarbonate, pH 6.0. The treated ACP (20 ml, 6 mg/ml) is
then mixed with 20ml of CNBr-activated Sepharose 4B swollen
in 0.1M sodium bicarbonate, pH 7.0, and the mixture is
20 allowed to stand at 4°C for one day. The gel suspension is
then centrifuged, washed once with 0.1M sodium bicarbonate,
pH 7.0, and then treated with 40ml 0.1M glycine, pH 8.0,
for 4 hours at room temperature to block unreacted sites.
The gel is then washed for five cycles with alternating
25 50ml volumes of 0.5M NaCl in 0.1M sodium acetate, pH 4.0,
and 0.5M NaCl in 0.1M sodium bicarbonate, pH 6.5, to remove
non-covalently bound ligand. The gel is loaded into a
column (1.5 x 11.2 cm) and equilibrated in 20mM potassium
phosphate, pH 6.8.
30   The combined fractions from the DE-52 column are
applied to the column, which is subsequently washed with
one column volume (20ml) of the equilibration buffer, and
then with 2.5 column volumes (50ml) of 300mM potassium
phosphate, pH 6.8. Fractions are assayed for protein using
35 the BCA Protein Assay Reagent (Pierce, Rockford, IL) to
make sure that all extraneous protein has been eluted.
Active Δ-9 desaturase is eluted from the column with 600mM
potassium phosphate, pH 6.8. Active fractions are analyzed

by polyacrylamide gel electrophoresis in sodium dodecyl
sulfate (SDS-PAGE) on 0.75mm thick 8 x 12 cm mini-gels
according to the method of Laemmli (*Nature* (1970) *227*:680).
The running gel contains 10% acrylamide in a 30/0.8 ratio

5   of acrylamide to cross-linker bis-acrylamide. Those
fractions containing a predominant band at approximately 43
kD are pooled and stored frozen at -70°C until final
purification. The yield from 50g of seed tissue is is
approximately 60μg of protein as measured by amino acid

10   analysis.
Further purification as described in Example 2 or
Example 3 is then applied to the fractions pooled from the
ACP-Sepharose column separation.

15   **Example 2**
In this example, a protocol for the final purification
of *C. tinctorius* desaturase is described. Seeds are
treated in accordance with Example 1.
Reverse-Phase HPLC: Fractions from the ACP-Sepharose

20   column are pooled and applied to a Vydac C4 reverse-phase
column (0.45 x 15 cm) equilibrated in 0.1% TFA, 7%
acetonitrile. After a 10 min wash with 0.1% TFA, the
column is eluted with a gradient of increasing acetonitrile
(7%-70% v/v) in 0.1% TFA over a period of 45 min. The flow

25   rate is 0.5ml/min throughout. Eluting components are
monitored by absorbance at 214 nm. Δ-9 desaturase elutes
at about 42 min. (approximately 50% acetonitrile); the
major contaminant protein remaining from ACP-affinity
chromatography elutes at about 28 min. (approximately 30%

30   acetonitrile). The substantially homogeneous desaturase,
which is no longer active, is identified by SDS-PAGE, in
which it exhibits a single band corresponding to a
molecular weight of approximately 43 kD. The quantity of
desaturase protein in the sample may be determined by amino

35   acid analysis.

Example 3

In this example, a protocol for the final purification of *C. tinctorius* desaturase is described. Seeds are treated in accordance with Example 1.

5   *Reduction and Alkylation:* Protein is precipitated out of the pooled fraction solutions recovered from the ACP-Sepharose column with 10% (w/v) trichloroacetic acid, washed with cold (-20°C) acetone, and resuspended in 1 ml 500mM Tris-HCl, pH 8.6, containing 6M guanidine-HCl, 10mM

10   EDTA, and 3.2 mM dithiothreitol. After 2 hours, 3.52 μmol [^{3}H]-iodoacetic acid (64μCi/μmol, New England Nuclear) is added, and the reaction is allowed to proceed at room temperature in the dark for 2 hours, at which time the reaction is terminated by addition of 1μl (15μmol) ß-

15   mercaptoethanol. The sample is then re-precipitated with 10% (w/v) trichloroacetic acid, and the pellet again washed with cold (-20°C) acetone and resuspended in Laemmli's SDS-sample buffer (*Nature* (1970) *227*:680).

SDS-Polyacrylamide Gel Electrophoresis: The resulting

20   sample is boiled for 5 min. and then applied to a 1.5 mm thick, 8 x 12 cm, SDS-polyacrylamide mini-gel prepared as described by Laemmli, *supra*. The running gel contains 17.5% acrylamide in a 30:0.13 ratio of acrylamide to cross-linking bis-acrylamide. Separation is achieved by

25   electrophoresis at 15 mA, for 2 hours at 4°C.

Blotting from SDS-gels to PVDF Membrane: Proteins are recovered from the gel by electroblotting at 5 mA/cm^2 to a four-layer sandwich of polyvinylidenedifluoride (PVDF) membrane for 2 h at 4°C in a buffer containing 10mM CAPS

30   ("3-[cyclohexylamino]-1-propane-sulfonic acid"), pH 11. The membranes must be wetted in 50% methanol, prior to exposure to the blotting buffer. After blotting, the membrane layers are stained for 1-2 min. in 0.02% Coomassie Blue in 50% methanol, and then destained in 50% methanol.

35   The desaturase is identified as a band corresponding to a molecular weight of about 43 kD; the major contaminant runs at or near the dye front of the gel corresponding to a molecular weight less than 20 kD.

The desaturase band on the PVDF membrane may be applied directly to the Edman sequencer (Applied Biosystems Model 477A) for determination of the N-terminal sequence of the intact protein, or for more extensive sequence
5   determination, may be eluted from the membrane in 40% acetonitrile to recover pure desaturase in solution. Acetonitrile is removed from the eluted desaturase by evaporation on a Speed-Vac (Savant; Farmingdale, NY), and the substantially homogeneous Δ-9 desaturase is resuspended
10 in an appropriate buffer for subsequent proteolytic digestion as described in Example 4. The quantity of desaturase protein present may be determined by amino acid analysis.

Alternatively, if the sample is to be digested with
15 trypsin or gluC protease to generate peptides for amino acid sequence analysis, proteins may be electroblotted to nitrocellulose membranes and stained with Ponceau or amido black.

20 **Example 4**

In this example, a method for the determination of the amino acid sequence of a desaturase is described.

Reduction and Alkylation: Substantially homogenous stearoyl-ACP desaturase (See, Example 2) is reduced and
25 alkylated with [3H]-iodacetic acid (See, Example 3), except that the final acetone-washed pellet is resuspended in the appropriate buffer for subsequent proteolysis. Reduction and alkylation assures complete denaturation of the protein so that complete proteolysis can occur. The sample may be
30 alkylated with radiolabeled iodoacetamide or with 4-vinylpyridine instead of [3H]-iodacetic acid in substantially the same manner. Use of iodoacetic acid affords an alkylated sample with greater solubility, which is advantageous in subsequent sample manipulation.

35 *Proteolysis:* Substantially pure alkylated samples are digested with endoproteinase lysC. The sample is resuspended in 100 μl of 25 mM Tris-HCl, pH 8.8, containing 1 mM EDTA. Endoproteinase lysC is added to the sample in a

protease/desaturase ratio of 1/50 (w/w). Digestion is
allowed to proceed at room temperature for 8 hours, at
which time another equal amount of protease is added.
After 18 more hours, 1 μl of concentrated HCl is added to
5   stop proteolysis, and the sample is applied directly to a
Vydac C18 reverse-phase column (0.2 x 15 cm) equilibrated
in 7% acetonitrile (v/v) in 0.1 mM sodium phosphate, pH
2.2. After washing for 20 min with the equilibration
buffer, peptides are eluted with a gradient in acetonitrile
10   (7-70%, v/v) over 120 min. Flow rate is 50 μl/min,
throughout. Eluting components are monitored by absorbance
at 214 nm, and individual peptide peaks are collected as
separate fractions. The peptide fractions are further
purified by application to a second Vydac C18 reverse-phase
15   column (0.2 x 15 cm) equilibrated in 7% (v/v) acetonitrile
in 0.1% (v/v) trifluoroacetic acid. Again, after a 20 min
wash with equilibration buffer, the substantially pure
peptides are eluted with a gradient (7-70%, v/v) of
acetonitrile in 0.1% trifluoroacetic acid over 120 min.
20   The flow rate is 50 μl/min, throughout. Eluting components
are monitored by absorbance at 214 nm, and individual
peptide peaks are collected as separate fractions. These
substantially pure peptides are applied directly to the
Edman sequencer (Applied Biosystems, Model 477A) for amino
25   acid sequence analysis. Alternatively, peptide fraction
from the first HPLC purification in phosphate buffer, or
from a single chromatography step in trifluoroacetic acid
buffer, may be applied directly to the sequencer, but these
fractions, in many cases, are not substantially pure and
30   yield mixed or ambiguous sequence information.

Other proteases may be used to digest desaturase,
including but not limited to trypsin, gluC, and aspN.
While the individual digest buffer conditions may be
different, the protocols for digestion, peptide separation,
35   purification, and sequencing are substantially the same as
those outlined for the digestion with lysC. Alternatively,
desaturase may be digested chemically using cyanogen
bromide (Gross *Methods Enzymol* (1967) *11*:238-255 or Gross

and Witkop *J. Am. Chem. Soc.* (1961) *83*:1510), hydroxylamine (Bornstein and Balian *Methods Enzymol.* (1977) *47*:132-745), iodosobenzoic acid (Inglis *Methods Enzymol.* (1983) *91*:324-332), or mild acid (Fontana *et al., Methods Enzymol.* (1983)
5   *91*:311-317), as described in the respective references.

Fragments generated from these digestion steps of *C. tinctorius* desaturase are presented in Fig. 1 and as SEQ ID NOS: 1-11.

10 **Example 5**

In this example, the preparation of a plant embryo cDNA bank, using the methods as described in Alexander, *et al.* (*Methods in Enzymology* (1987) *154*:41-64) and the screening of the bank to obtain a desaturase cDNA clone is
15 described.

C. tinctorius: A plant embryo cDNA library may be constructed from poly(A)+ RNA isolated from *C. tinctorius* embryos collected at 14-17 days post-anthesis. Poly(A)+ RNA is isolated from polyribosomes by a method initially
20 described by Jackson and Larkins (*Plant Physiol.* (1976) *57*:5-10) as modified by Goldberg *et al.* (*Developmental Biol.* (1981) *83*:201-217).

The plasmid cloning vector pCGN1703, derived from the commercial cloning vector Bluescribe M13- (Stratagene
25 Cloning Systems; San Diego, CA), is made as follows. The polylinker of Bluescribe M13- is altered by digestion with *Bam*HI, treatment with mung bean endonuclease, and blunt-end ligation to create a *Bam*HI-deleted plasmid, pCGN1700. pCGN1700 is digested with *Eco*RI and *Sst*I (adjacent
30 restriction sites) and annealed with synthetic complementary oligonucleotides having the sequences 5' CGGATCCACTGCAGTCTAGAGGGCCCGGGA 3'(SEQ ID NO: 30) and 5' AATTTCCCGGGCCCTCTAGACTGCAGTGGATCCGAGCT 3' (SEQ ID NO: 31). These sequences are inserted to eliminate the *Eco*RI
35 site, move the *Bam*HI site onto the opposite side of the *Sst*I (also, sometimes referred to as "*SacI*" herein) site found in Bluescribe, and to include new restriction sites *Pst*I, *Xba*I, *Apa*I, *Sma*I. The resulting plasmid pCGN1702, is

34

digested with *HindIII* and blunt-ended with Klenow enzyme; the linear DNA is partially digested with *PvuII* and ligated with T4 DNA ligase in dilute solution. A transformant having the *lac* promoter region deleted is selected

5   (pCGN1703) and is used as the plasmid cloning vector.

Briefly, the cloning method for cDNA synthesis is as follows. The plasmid cloning vector is digested with *SstI* and homopolymer T-tails are generated on the resulting 3'-overhang sticky-ends using terminal deoxynucleotidyl

10 transferase. The tailed plasmid is separated from undigested or un-tailed plasmid by oligo(dA)-cellulose chromatography. The resultant vector serves as the primer for synthesis of cDNA first strands covalently attached to either end of the vector plasmid. The cDNA-mRNA-vector

15 complexes are treated with terminal transferase in the presence of deoxyguanosine triphosphate, generating G-tails at the ends of the cDNA strands. The extra cDNA-mRNA complex, adjacent to the *BamHI* site, is removed by *BamHI* digestion, leaving a cDNA-mRNA-vector complex with a *BamHI*

20 sticky-end at one end and a G-tail at the other. This complex is cyclized using the annealed synthetic cyclizing linker,   5'-
GATCCGCGGCCGCGAATTCGAGCTCCCCCCCCCC-3' and
        3'-GCGCCGGCGCTTAAGCTCGA-5'

25 which has a *BamHI* sticky-end and a C-tail end. Following ligation and repair the circular complexes are transformed into *E. coli* strain DH5α (BRL; Gaithersburg, MD) to generate the cDNA library. The *C. tinctorius* embryo cDNA bank contains between 3x10^6 and 5x10^6 clones with an average

30 cDNA insert size of approximately 1000 base pairs.

Probe production Including PCR Reactions: Two regions of amino acid sequence (Example 4) with low codon degeneracy are chosen from opposite ends of peptide sequence "Fragment F2" (SEQ ID NO:2) for production of a

35 probe for the plant desaturase cDNA. Two sets of mixed oligonucleotides are designed and synthesized for use as forward (SEQ ID NOS: 21-24) and reverse (SEQ ID NOS: 25-26) primers, respectively, in the polymerase chain reaction

(Saiki et *al.*, *Science* (1985) *230*:1350-1354; Oste,
Biotechniques (1988) *6*:162-167). See, Fig. 6. All
oligonucleotides are synthesized on an Applied Biosystems
380A DNA synthesizer.

5   Probes to *C. tinctorius* desaturase may be prepared
using the peptide sequence "Fragment 2" (SEQ ID NO: 2)
identified in Fig. 1. Four types of forward primers were
synthesized and labeled 13-1, 13-2, 13-3, and 13-4 (SEQ ID
NOS: 21-24, respectively). Two groups of reverse primers

10   were synthesized and designated 13-5A and 13-6A (SEQ ID
NOS: 25-26, respectively). The primer sequences are shown
in Fig. 6. These oligonucleotide groups have a redundancy
of 64 or less and contain either 20 or 17 bases of coding
sequence along with flanking restriction site sequences for

15   *Hind*III or *Eco*RI. Based on the intervening amino acid
sequence between the primer regions on peptide "Fragment 2"
(SEQ ID NO: 2) the PCR product is expected to contain 107
base pairs.

       Polymerase chain reaction is performed using the cDNA

20   library DNA as template and the possible eight combinations
of the four forward and two reverse oligonucleotides as
primers in a Perkin-Elmer/Cetus DNA Thermal Cycler
(Norwalk, CT) thermocycle file 1 min. 94°C, 2 min. 42°C, 2
min rise from 42°-72°C for 30 cycles, followed by the step

25   cycle file without step rises, 1 min. 94°C, 2 min. 42°C, 3
min. 72°C with increasing 15 sec extensions of the 72°C
step for 10 cycles, and a final 10 min. 72°C extension.

       The product of the 13-4 forward primer (SEQ ID NO: 24)
and the 13-5A reverse primer (SEQ ID NO: 25) reaction was

30   ethanol precipitated and then digested with *Hind*III and
*Eco*RI, the resulting fragment was subcloned into pUC8
(Vieira and Messing, *Gene* (1982) *19*:259-268).
Minipreparation DNA (Maniatis et *al.*, *Molecular_Cloning: A
Laboratory Manual* (1982) Cold Harbor Laboratory, New York)

35   of one clone was sequenced by Sanger dideoxy sequencing
(Sanger et *al.*, *Proc. Nat. Acad. Sci. USA* (1977) 74:5463-
5467) using the M13 universal and reverse primers.
Translation of the resulting DNA sequence results in a

peptide sequence that exactly matches the amino acid
sequence in peptide "Fragment F2" (SEQ ID NO: 2).

     An exact 50 base oligonucleotide designated DESAT-50
is synthesized to match the sequence of the PCR reaction
5 product from the first valine residue to the last tyrosine
residue.

     The probe DSAT-50 5' -
GTAAGTAGGTAGGGCTTCCTCTGTAATCATATCTCCAACCAAAACAACAA -3' (SEQ
ID NO: 32) is used to probe the *C. tinctorius* embryo cDNA
10 library.

Library screen

     The *C. tinctorius* embryo cDNA bank is moved into the
cloning vector lambda gt10 (Stratagene Cloning Systems) by
15 digestion of total cDNA with *EcoRI* and ligation to lambda
gt10 DNA digested with *EcoRI*. The titer of the resulting
library was ~5x10^5/ml. The library is then plated on *E.
coli* strain C600 (Huynh, et al., *DNA Cloning Vol. 1* Eds.
Glover D.M. IRL Press Limited: Oxford England, pp. 56, 110)
20 at a density of 5000 plaques/150 mm NZY ("NZYM" as defined
in Maniatis et al. *supra*) agar plate to provide over 45,000
plaques for screening. Duplicate lifts are taken of the
plaques using NEN Colony Plaque Screen filters by laying
precut filters over the plates for ~1 minute and then
25 peeling them off. The phage DNA is immobilized by floating
the filters on denaturing solution (1.5M NaCl, .05M NaOH)
for 1 min., transferring the filters to neutralizing
solution (1.5M NaCl, 0.5M Tris-HCI pH 8.0) for 2 min. and
then to 2XSSC (1xSSC = 0.15M NaCl; 0.015M Na citrate) for 3
30 min., followed by air drying. The filters are hybridized
with ^{32}P end-labeled DSAT-50 oligonucleotide (SEQ ID NO:
32) (BRL 5' DNA Terminus Labeling System) by the method of
Devlin et al., (*DNA* (1988) 7:499-807) at 42° C overnight,
and washed for 30 min. at 50°C in 2XSSC, 0.5% SDS and then
35 twice for 20 min. each at 50°C in 0.1XSSC, 0.5% SDS.
Filters are exposed to X-ray film at -70°C with a Dupont
Cronex intensifying screen for 48 hours.

Clones are detected by hybridization with the DSAT-50
oligonucleotide and plaque purified. The complete
nucleotide sequence (SEQ ID NO: 12) of the cDNA insert of a
clone, pCGN2754, and a partial restriction map thereof are
5   presented in Figures 2 and 7A, respectively. The cDNA
insert includes 1533 bases plus a poly(A) track at the 3'
end of 100-200 bases. The open reading frame for the
desaturase begins at the first ATG (nucleotide 106) from
the 5' end and stops at nucleotide 1294. The translated
10   amino acid sequence is presented in Fig. 2 and SEQ ID NO:
13. The open reading frame includes a 33 amino acid
transit peptide not found in the amino acid sequence of the
mature protein. The N-terminus of the protein begins at
the alanine immediately following the NcoI site (nucleotide
15   202) indicating the site of the transit peptide processing.

Example 6

In this example, expression of a plant desaturase in a
prokaryote is described.
20   *Desaturase expression construct in E. coli*

A plasmid for expression of desaturase activity in *E.
coli* is constructed as follows. The desaturase cDNA clone
pCGN2754 is digested with HindIII and SalI and ligated to
pCGN2016 (a chloramphenicol resistant version of Bluescript
25   KS-) digested with HindIII and XhoI. The resulting plasmid
is pCGN1894.

pCGN2016 is prepared by digesting pCGN565 with HhaI,
and the fragment containing the chloramphenicol resistance
gene is excised, blunted by use of mung bean nuclease, and
30   inserted into the EcoRV site of Bluescript KS- (Stratagene:
La Jolla, CA) to create pCGN2008. The choramphenicol
resistance gene of pCGN2008 is removed by EcoRI/HindIII
digestion. After treatment with Klenow enzyme to blunt the
ends, the fragment is ligated to DraI digested Bluescript
35   KS-. A clone that has the DraI fragment containing
ampicillin resistance replaced with the chloramphenicol
resistance is chosen and named pCGN2016.

pCGN565 is a cloning vector based on pUC12-cm (K. Buckley
Ph.D. Thesis, Regulation and expression of the phi X174 lysis
gene, University of California, San Diego, 1985), but contains
pUC18 linkers (Yanisch-Perron, *et al.*, *Gene* (1985) *53*:103-119).

5   The fragment containing the mature coding region of
the Δ-9 desaturase, 3'-noncoding sequences and poly(A)
tails is subcloned from pCGN1894 digested with *Nco*1 and
*Asp*718 into pUC120, an *E. coli* expression vector based on
pUC118 (Vieira and Messing, *Methods in Enzymology* (1987)

10   *153*:3-11) with the lac region inserted in the opposite
orientation and an *Nco*I site at the ATG of the lac peptide
(Vieira, J. PhD. Thesis, University of Minnesota, 1988).
The *E. coli* desaturase expression plasmid is designated
pCGN3201. The desaturase sequences are inserted such that

15   they are aligned with the lac transcription and translation
signals.

Expression of Desaturase in E.coli
       Single colonies of *E. coli* strain 7118 (Maniatis *et*

20   *al.*, *supra*) containing pUC120 or pCGN3201 are cultured in
80 mls each of ECLB broth, 300 mg/L penicillin. The cells
are induced by the addition of 1mM IPTG. Cells are grown
overnight (18 hrs) at 37° C.
       Eighty mls of overnight cultures of *E. coli* (induced

25   and uninduced) containing pUC120 or pCGN3201 are
centrifuged at 14,800 x g for 15 min. The pelleted cells
are resuspended in 3 mls 20 mM phosphate buffer, pH 6.8.
Resuspended cells were broken in a french press at 16,000
psi. Broken cell mixtures are centrifuged 5000xg for 5

30   min. 100 μl of each supernatant is applied to a G-25
Sephadex gel filtration centrifugal column (Boehringer
Mannheim Biochemicals), equilibrated in 20mM phosphate
buffer pH 6.8. Columns are spun for 4 min at 5000xg.
Effluent was collected and used as enzyme source in the

35   desaturase assay.
       Desaturase activity is assayed as described in Example
1. Both pUC120-containing, IPTG-induced cells and
uninduced cells do not express detectable stearoyl-ACP

desaturase activity. The pCGN3201 IPTG-induced extract
contains 8.22 nmol/min of desaturase activity. pCGN3201
uninduced extracts contains 6.45 nmol/min of activity. The
pCGN3201 IPTG-induced extract shows 21.5% more activity
5   than the uninduced pCGN3201 extract.

Detection of induced protein in E. coli.

Extracts of overnight cultures of *E. coli* strain 7118
(Maniatis *et al. supra*) containing pCGN3201 or pUC120
10 grown in ECLB containing 300 mg/L penicillin induced with
1mM IPTG are prepared as follows. 1.5 ml of overnight
culture grown shaking at 37°C are pelleted in Eppendorf
tubes for 10 min at 10-13,000 μg. Pellets are resuspended
in 150 ul SDS sample buffer (0.05M Tris-HCl, pH6.8, 1% SDS,
15 5% ß-mercaptoethanol, 10% glycerol and 0.005% bromophenol
blue) and boiled for 10 min. 25 μl of each sample are
electrophoresed on a 10% polyacrylamide gel (Laemmli,
Nature (1970) *227*:680) at 25 mA for 5 hours. Gels are
stained in 0.05% Coomassie Brilliant Blue, 25% isopropanol
20 and 10% acetic acid and destained in 10% acetic acid and
10% isopropanol. A band is detected at a position just
below the 43,000 MW protein marker (SDS PAGE standard, Low
molecular weight, BioRad, Richmond CA) in the pCGN3201
extracts that is not present in the pUC120 extracts. This
25 is the approximate molecular weight of mature desaturase
protein.

Requirement for Spinach Ferredoxin

Stearoyl-ACP desaturase can also be expressed in *E.*
30 *coli* by subcloning into the *E. coli* expression vector
pET8c (Studier, *et al., Methods Enzymol.* (1990) *185*:60-89).
The mature coding region (plus an extra Met codon) of the
desaturase cDNA with accompanying 3'-sequences is inserted
as an *Nco*1 - *Sma* 1 fragment into pET8c at the *Nco*1 and
35 *Bam*H1 sites (after treatment of the *Bam*H1 site with Klenow
fragment of DNA polymerase to create a blunt end) to create
pCGN3208. The plasmid pCGN3208 is maintained in *E. coli*
strain BL21(DE3) which contains the T7 polymerase gene

under the control of the isopropyl-b-D-
thiogalactopyranoside (IPTG)-inducible *lac*UV5 promoter
(Studier et *al.*, *supra*).

5   *E. coli* cells containing pCGN3208 are grown at 37°C to
an OD$_{595}$ of ~0.7 in NZY broth containing 0.4% (w/v) glucose
and 300 mg/liter penicillin, and then induced for 3 hours
with 0.4 mM IPTG. Cells are pelleted from 1 ml of culture,
dissolved in 125 μl of SDS sample buffer (10) and heated to
100°C for 10 min. Samples are analyzed by SDS
10   polyacrylamide gel electrophoresis at 25 mA for 5 h. Gels
are stained in 0.05% Coomassie Brilliant Blue, 25% (v/v)
isopropanol and 10% (v/v) acetic acid. A band is detected
at a position just below the 43,000 MW protein marker (*SDS*
PAGE standard, Low Molecular Weight, BioRad, Richmond, CA)
15   in the pCGN3208 extract that is not present in the pET8c
extracts. This is the approximate molecular weight of
mature desaturase protein.

For activity assays, cells are treated as described
above and extracts are used as enzyme source in the
20   stearoyl-ACP desaturase assay as described in Example 1.
The extract from IPTG-induced pCGN3208 cells contains 8.61
nmol/min/mg protein of desaturase activity. The extract
from pCGN3208 uninduced cells contains 1.41 nmol/min/mg
protein of desaturase activity. The extract from pCGN3208
25   induced cells, thus has approximately 6-fold greater
activity than the extract from uninduced pCGN3208 cells.
Extracts from both induced and uninduced cells of pET8c do
not contain detectable stearoyl-ACP desaturase activity.

Samples are also assayed as described in Example 1,
30   but without the addition of spinach ferredoxin, to
determine if the *E. coli* ferredoxin is an efficient
electron donor for the desaturase reaction. Minimal
activity is detected in *E. coli* extracts unless spinach
ferredoxin is added exogenously.

35

Example 7

In this example, the preparation of an ACP expression cassette containing a plant desaturase in a binary vector suitable for plant transformation is described.

5

ACP Expression Cassette

An expression cassette utilizing 5'-upstream sequences and 3'-downstream sequences obtainable from *B. campestris* ACP gene can be constructed as follows.

10   A 1.45kb *XhoI* fragment of Bcg 4-4 (Fig. 9 and SEQ ID NO: 28) containing 5'-upstream sequences is subcloned into the cloning/sequencing vector Bluescript+ (Stratagene Cloning Systems, San Diego, CA). The resulting construct, pCGN1941, is digested with *XhoI* and ligated to a

15   chloramphenicol resistant Bluescript M13+ vector, pCGN2015 digested with *XhoI*. pCGN2015 is prepared as described for pCGN2016 (See, Example 6) except that the *EcoRI/HindIII* "chloramphenicol" fragment isolated from pCGN2008 is ligated with the 2273 bp fragment of Bluescript KS+

20   (Stratagene; LaJolla, CA) isolated after digestion with *DraI*. This alters the antibiotic resistance of the plasmid from penicillin resistance to chloramphenicol resistance. The chloramphenicol resistant plasmid is pCGN1953.

        3'-sequences of Bcg 4-4 are contained on an *SstI/BglII*

25   fragment cloned in the *SstI/BamHI* sites of M13 Bluescript+ vector. This plasmid is named pCGN1940. pCGN1940 is modified by *in vitro* site-directed mutagenesis (Adelman et al., *DNA* (1983) 2:183-193) using the synthetic oligonucleotide 5'-CTTAAGAAGTAACCCGGGCTGCAGTTTTAGTATTAAGAG-

30   3' (SEQ ID NO: 33) to insert *SmaI* and *PstI* restriction sites immediately following the stop codon of the reading frame for the ACP gene 18 nucleotides from the *SstI* site. The 3'-noncoding sequences from this modified plasmid, pCGN1950, are moved as a *PsI-SmaI* fragment into pCGN1953

35   cut with *PstI* and *SmaI*. The resulting plasmid pCGN1977 comprises the ACP expression cassette with the unique restriction sites *EcoRV*, *EcoRI* and *PstI* available between the 1.45kb 5' and 1.5 kb of 3'-noncoding sequences (SEQ ID

42

NO: 28) for the cloning of genes to be expressed under regulation of these ACP gene regions.

Desaturase Expression in Plants

Desaturase cDNA sequences from pCGN2754 are inserted in the ACP expression cassette, pCGN1977, as follows. pCGN2754 is digested with *Hin*dIII (located 160 nucleotides upstream of the start codon) and *Asp*718 located in the polylinker outside the poly(A) tails. The fragment containing the coding region for desaturase was blunt-ended using DNA polymerase I and ligated to pCGN1977 digested with *Eco*RV. A clone containing the desaturase sequences in the sense orientation with respect to the ACP promoter is selected and called pCGN1895. This expression cassette may be inserted into a binary vector, for example, for *Agrobacterium*-mediated transformation, or employed in other plant transformation techniques.

Binary Vector and Agrobacterium Transformation

The fragment containing the pCGN1895 expression sequences ACP 5'/desaturase/ACP 3' is cloned into a binary vector pCGN1557 (described below) for *Agrobacterium* transformation by digestion with *Asp*718 and *Xba*I and ligation to pCGN1557 digested with *Asp*718 and *Xba*I. The resulting binary vector is called pCGN1898.

pCGN1898 is transformed into *Agrobacterium tumefaciens* strain EHA101 (Hood, et *al.*, *J. Bacteriol.* (1986) *168*:1291-1301) as per the method of Holsters, et *al.*, *Mol. Gen. Genet.* (1978) *163*:181-187.

RNA blot analysis of seeds (T2) from T1 plants show the presence of a mRNA species for the inserted *C. tinctorius* desaturase, but the amount of message is low compared to endogenous levels of mRNA for the *Brassica* desaturase, suggesting that the expression levels were insufficient to significantly increase the amount of desaturase enzyme above that normally present. This is consistent with the negative results from oil, desaturase activity and Western blot analyses.

Construction of pCGN1557

pCGN1557 (McBride and Summerfelt, *Plant Molecular Biology* (1990) *14(2)*:269-276) is a binary plant
5 transformation vector containing the left and right T-DNA borders of *Agrobacterium tumefaciens* octopine Ti-plasmid pTiA6 (Currier and Nester, *supra*, the gentamycin resistance gene of pPH1JI (Hirsch and Beringer, *supra*), an *Agrobacterium rhizogenes* Ri plasmid origin of replication
10 from pLJbB11 (Jouanin et *al.*, *supra*), a 35S promoter-kanR-tml3' region capable of conferring kanamycin resistance to transformed plants, a ColE1 origin of replication from pBR322 (Bolivar et *al.*, *supra*), and a *lacZ'* screenable marker gene from pUC18 (Yanish-Perron et *al.*, *supra*).
15 There are three major intermediate constructs used to generate pCGN1557:

pCGN1532 (see below) contains the pCGN1557 backbone, the pRi plasmid origin of replication, and the ColE1 origin of replication.
20 pCGN1546 (see below) contains the CaMV35S5'-kan^R-tml3' plant selectable marker region.

pCGN1541b (see below) contains the right and left T-DNA borders of the *A. tumefaciens* octopine Ti-plasmid and the *lacZ'* region from pUC19.
25 To construct pCGN1557 from the above plasmids, pCGN1546 is digested with *Xho*I, and the fragment containing the CaMV 35S5'-kan^R-tml3' region is cloned into the *Xho*I site of pCGN1541b to give the plasmid pCGN1553, which contains T-DNA/left border/CaMV 35S5'-kanR-tml3'/*lacZ'*/T-
30 DNA left border. pCGN1553 is digested with *Bgl*II, and the fragment containing the T-DNA/left border/CaMV35S5'-kan^R- tml3'/*lacZ'*/T-DNA left border region is ligated into *Bam*HI-digested pCGN1532 to give the complete binary vector, pCGN1557.
35 pCGN1532

The 3.5kb *Eco*RI-*Pst*I fragment containing the gentamycin resistance gene is removed from pPh1JI (Hirsch and Beringer, *Plasmid* (1984) *12*:139-141) by *Eco*RI-*Pst*I

44

digestion and cloned into *EcoRI-PstI* digested pUC9 (Vieira
and Messing, *Gene* (1982) *19*:259-268) to generate pCGN549.
HindIII-PstI digestion of pCGN549 yields a 3.1 kb fragment
bearing the gentamycin resistance gene, which is made blunt

5 ended by the Klenow fragment of DNA polymerase I and cloned
into *PvuII* digested pBR322 (Bolivar et *al., Gene* (1977)
2:95-113) to create pBR322Gm. pBR322Gm is digested with
DraI and *SphI*, treated with Klenow enzyme to create blunt
ends, and the 2.8 kb fragment cloned into the Ri origin-

10 containing plasmid pLJbB11 (Jouanin et *al., Mol. Gen.
Genet.* (1985) *201*:370-374) which has been digested with
ApaI and made blunt-ended with Klenow enzyme, creating
pLHbB11Gm. The extra ColE1 origin and the kanamycin
resistance gene are deleted from pLHbB11Gm by digestion

15 with *BamHI* followed by self closure to create pGmB11. The
HindII site of pGmB11 is deleted by *HindIII* digestion
followed by treatment with Klenow enzyme and self closure,
creating pGmB11-H. The *PstI* site of pGmB11-H is deleted by
PstI digestion followed by treatment with Klenow enzyme and

20 self closure, creating pCGN1532.

Construction of pCGN1546

The 35S promoter-tml3' expression cassette, pCGN986,
contains a cauliflower mosaic virus 35S (CaMV35) promoter

25 and a T-DNA tml 3'-region with multiple restriction sites
between them. pCGN986 is derived from another cassette,
pCGN206, containing a CaMV35S promoter and a different 3'
region, the CaMV region VI 3'-end. The CaMV 35S promoter
is cloned as an *AluI* fragment (bp 7144-7734) (Gardner et.

30 *al., Nucl. Acids Res.* (1981) *9*:2871-2888) into the *HincII*
site of M13mp7 (Messing, et. *al., Nucl. Acids Res.* (1981)
9:309-321) to create C614. An *EcoRI* digest of C614
produced the *EcoRI* fragment from C614 containing the 35S
promoter which is cloned into the *EcoRI* site of pUC8

35 (Vieira and Messing, *Gene* (1982) *19*:259) to produce
pCGN147.

pCGN148a containing a promoter region, selectable
marker (KAN with 2 ATG's) and 3' region, is prepared by

digesting pCGN528 with *BglII* and inserting the *BamHI-BglII*
promoter fragment from pCGN147. This fragment is cloned
into the *BglII* site of pCGN528 so that the *BglII* site is
proximal to the kanamycin gene of pCGN528.

5   The shuttle vector used for this construct pCGN528, is
made as follows: pCGN525 is made by digesting a plasmid
containing Tn5 which harbors a kanamycin gene (Jorgenson
et. al., Mol. Gen. Genet. (1979) *177*:65) with *HindIII-BamHI*
and inserting the *HindIII-BamHI* fragment containing the
10   kanamycin gene into the *HindIII-BamHI* sites in the
tetracycline gene of pACYC184 (Chang and Cohen, *J.
Bacteriol.* (1978) *134*:1141-1156). pCGN526 was made by
inserting the *BamHI* fragment 19 of pTiA6 (Thomashow *et.
al., Cell* (1980) *19*:729-739), modified with *XhoI* linkers
15   inserted into the *SmaI* site, into the *BamHI* site of
pCGN525. pCGN528 is obtained by deleting the small *XhoI*
fragment from pCGN526 by digesting with *XhoI* and
religating.

        pCGN149a is made by cloning the *BamHI*-kanamycin gene
20   fragment from pMB9KanXXI into the *BamHI* site of pCGN148a.
pMB9KanXXI is a pUC4K variant (Vieira and Messing, *Gene*
(1982) *19*:259-268) which has the *XhoI* site missing, but
contains a functional kanamycin gene from Tn903 to allow
for efficient selection in *Agrobacterium*.

25   pCGN149a is digested with *HindIII* and *BamHI* and
ligated to pUC8 digested with *HindIII* and *BamHI* to produce
pCGN169. This removes the Tn903 kanamycin marker. pCGN565
(see pCGN2016 description) and pCGN169 are both digested
with *HindIII* and *PstI* and ligated to form pCGN203, a
30   plasmid containing the CaMV 35S promoter and part of the
5'-end of the Tn5 kanamycin gene (up to the *PstI* site,
Jorgenson *et. al.,* (1979), *supra*). A 3'-regulatory region
is added to pCGN203 from pCGN204 (an *EcoRI* fragment of CaMV
(bp 408-6105) containing the region VI 3' cloned into pUC18
35   (Yanisch-Perron, *et al., Gene* (1985) *33*:103-119) by
digestion with *HindIII* and *PstI* and ligation. The
resulting cassette, pCGN206, is the basis for the
construction of pCGN986.

The pTiA6 T-DNA tml 3'-sequences are subcloned from
the *Bam*19 T-DNA fragment (Thomashow *et al.*, (1980) *supra*)
as a *Bam*HI-*Eco*RI fragment (nucleotides 9062 to 12,823,
numbering as in Barker *et al.*, *Plant Mol. Biol.* (1982)

5   *2*:335-350) and combined with the pACYC184 (Chang and Cohen
(1978), *supra*) origin of replication as an *Eco*RI-*Hind*III
fragment and a gentamycin resistance marker (from plasmid
pLB41), obtained from D. Figurski) as a *Bam*HI-*Hind*III
fragment to produce pCGN417.

10   The unique *Sma*I site of pCGN417 (nucleotide 11,207 of
the *Bam*19 fragment) is changed to a *Sac*I site using linkers
and the *Bam*HI-*Sac*I fragment is subcloned into pCGN565 to
give pCGN971. The *Bam*HI site of pCGN971 is changed to an
*Eco*RI site using linkers. The resulting *Eco*RI-*Sac*I

15 fragment containing the tml 3' regulatory sequences is
joined to pCGN206 by digestion with *Eco*RI and *Sac*I to give
pCGN975. The small part of the Tn5 kanamycin resistance
gene is deleted from the 3'-end of the CaMV 35S promoter by
digestion with *Sal*I and *Bgl*II, blunting the ends and

20 ligation with *Sal*I linkers. The final expression cassette
pCGN986 contains the CaMV 35S promoter followed by two *Sal*I
sites, an *Xba*I site, *Bam*HI, *Sma*I, *Kpn*I and the tml 3'
region (nucleotides 11207-9023 of the T-DNA).

        The 35S promoter-tml 3' expression cassette, pCGN986

25 is digested with *Hind*III. The ends are filled in with
Klenow polymerase and *Xho*I linkers added. The resulting
plasmid is called pCGN986X. The *Bam*HI-*Sac*I fragment of
pBRX25 (see below) containing the nitrilase gene is
inserted into *Bam*HI-*Sac*I digested pCGN986X yielding pBRX66.

30   Construction of pBRX25 is described in U.S. Letters
Patent 4,810,648, which is hereby incorporated by
reference. Briefly, the method is as follows: The
nucleotide sequence of a 1212-bp *Pst*I-*Hinc*II DNA segment
encoding the bromoxynil-specific nitrilase contains 65-bp

35 of 5' untranslated nucleotides. To facilitate removal of a
portion of these excess nucleotides, plasmid pBRX9 is
digested with *Pst*I, and treated with nuclease *Bal*31. *Bam*HI
linkers are added to the resulting ends. *Bam*HI-*Hinc*II

47

fragments containing a functional bromoxynil gene are cloned into the *Bam*HI-*Sma*I sites of pCGN565. The resulting plasmid, pBRX25, contains only 11 bp of 5' untranslated bacterial sequence.

5   pBRX66 is digested with *Pst*I and *Eco*RI, blunt ends generated by treatment with Klenow polymerase, and *Xho*I linkers added. The resulting plasmid pBRX68 now has a tml 3' region that is approximately 1.1kb. pBRX68 is digested with *Sal*I and *Sac*I, blunt ends generated by treatment with 10   Klenow polymerase and *Eco*RI linkers added. The resulting plasmid, pCGN986XE is a 35S promoter - tml 3' expression cassette lacking the nitrilase gene.

       The Tn5 kanamycin resistance gene is then inserted into pCGN986XE. The 1.0 kb *Eco*RI fragment of pCGN1536 (see 15   pCGN1547 description) is ligated into pCGN986XE digested with *Eco*RI. A clone with the Tn5 kanamycin resistance gene in the correct orientation for transcription and translation is chosen and called pCGN1537b. The 35S promoter Kan^R-tml 3' region is then transferred to a 20   chloramphenical resistant plasmid backbone. pCGN786, (a pUC-CAM based vector with the synthetic oligonucleotide 5' GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3' (SEQ ID NO: 34) containing the cloning sites *Eco*RI, *Sal*I, *Bgl*II, *Pst*I, *Xho*I, *Bam*HI, and *Hin*dIII inserted into pCGN566, pCGN566 25   contains the *Eco*HI-*Hin*dIII linker of pUC18 inserted into the *Eco*KI-*Hin*dIII sites of pUC13-cm (K. Buckler (1985) *supra*)) is digested with *Xho*I and the *Xho*I fragment of pCGN1537b containing the 35S promoter - Kan^R-tml 3' region is ligated in. The resulting clone is termed pCGN1546.

30

pCGN1541b

       pCGN565RBα2X (see below) is digested with *Bgl*II and *Xho*I, and the 728bp fragment containing the T-DNA right border piece and the *lacZ'* gene is ligated with *Bgl*II-*Xho*I 35   digested pCGN65ΔKX-S+K (see below), replacing the *Bgl*II-*Xho*I right border fragment of pCGN65ΔKX-S+K. The resulting plasmid, pCGN65α2X contains both T-DNA borders and the *lacZ'* gene. The *Cla*I fragment of pCGN65α2X is

48

replaced with an *XhoI* site by digesting with *ClaI* blunting the ends using the Klenow fragment, and ligating with *XhoI* linker DNA, resulting in plasmid pCGN65α2XX. pCGN65α2XX

is digested with *BglII* and *EcoRV*, treated with the Klenow
5   fragment of DNA polymerase I to create blunt ends, and ligated in the presence of *BglII* linker DNA, resulting in pCGN65α2XX'. pCGN65α2XX' is digested with *BglII* and ligated with *BglII* digested pCGN1538 (see below), resulting in pCGN1541a, which contains both plasmid backbones.
10 pCGN1541a is digested with *XhoI* and religated. Ampicillin resistant, chlormaphenicol sensitive clones are chosen, which lack the pACYC184-derived backbone, creating pCGN1541b.

   pCGN1538 is generated by digesting pBR322 with *EcoRI*
15 and *PvuII*, treating with Klenow to generate blunt ends, and ligating with *BglII* linkers. pCGN1538 is ampicillin resistant, tetracycline sensitive.

   pCGN65ΔKX-S+K
20   pCGN501 is constructed by cloning a 1.85 kb *EcoRI-XhoI* fragment of pTiA6 (Currier and Nester, *J. Bact.* (1976) *126*:157-165) containing bases 13362-15208 (Barker et *al.*, *Plant Mo. Biol.* (1983) *2*:335-350) of the T-DNA (right border), into *EcoRI-SalI* digested M13mp9 (Vieira and
25 Messing, *Gene* (1982) *19*:259-268). pCGN502 is constructed by cloning a 1.6 kb *HindIII-SmaI* fragment of pTiA6, containing bases 602-2212 of the T-DNA (left border), into *HindIII-SmaI* digested M13mp9. pCGN501 and pCGN502 are both digested with *EcoRI* and *HindIII* and both T-DNA-containing
30 fragments cloned together into *HindIII* digested pUC9 (Vieira and Messing, *Gene* (1982) *19*:259-268) to yield pCGN503, containing both T-DNA border fragments. pCGN503 is digested with *HindIII* and *EcoRI* and the two resulting *HindIII-EcoRI* fragments (containing the T-DNA borders) are
35 cloned into *EcoRI* digested pHC79 (Hohn and Collins, *Gene* (1980) *11*:291-298) to generate pCGN518. The 1.6kb *KpnI-EcoRI* fragment from pCGN518, containing the left T-DNA border, is cloned into *KpnI-EcoRI* digested pCGN565 to

generate pCGN580. The *BamH*II-*Bgl*II fragment of pCGN580 is
cloned into the *Bam*HI site of pACYC184 (Chang and Cohen, *J.
Bacteriol.* (1978) *134*:1141-1156) to create pCGN51. The 1.4
kb *Bam*HI-*Sph*I fragment of pCGN60 containing the T-DNA right
5   border fragment, is cloned into *Bam*HI-*Sph*I digested pCGN51
to create pCGN65, which contains the right and left T-DNA
borders.

pCGN65 is digested with *Kpn*I and *Xba*I, treated with
Klenow enzyme to create blunt ends, and ligated in the
10 presence of synthetic *Bgl*II linker DNA to create pCGN65ΔKX.
pCGN65ΔKX is digested with *Sal*I, treated with Klenow enzyme
to create blunt ends, and ligated in the presence of
synthetic *Xho*I linker DNA to create pCGN65ΔKX-S+X.

15   <u>pCGN565RBα2X</u>

pCGN451 (see below) is digested with *Hpa*I and ligated
in the presence of synthetic *Sph*I linker DNA to generate
pCGN55. The *Xho*I-*Sph*I fragment of pCGN55 (bp13800-15208,
including the right border, of *Agrobacterium tumefaciens* T-
20 DNA; (Barker *et al.*, *Gene* (1977) *2*:95-113) is cloned into
*Sal*I-*Sph*I digested pUC19 (Yanisch-Perron *et al.*, *Gene*
(1985) *53*:103-119) to create pCGN60. The 1.4 kb *Hind*III-
*Bam*HI fragment of pCGN60 is cloned into *Hind*III-*Bam*HI
digested pSP64 (Promega, Inc.) to generate pCGN1039.
25 pCGN1039 is digested with *Sma*I and *Nru*I (deleting bp14273-
15208; (Barker *et al.*, *Gene* (1977) *2*:95-113) and ligated in
the presence of synthetic *Bgl*II linker DNA creating
pCGN1039ΔNS. The 0.47 kb *Eco*RI-*Hind*III fragment of
pCGN1039ΔNS is cloned into *Eco*RI-*Hind*III digested pCGN565
30 to create pCGN565RB. The *Hind*III site of pCGN565RB is
replaced with an *Xho*I site by digesting with *Hind*III,
treating with Klenow enzyme, and ligating in the presence
of synthetic *Xho*I linker DNA to create pCGN565RB-H+X.

pUC18 (Norrander *et al.*, *Gene* (1983) *26*:101-106) is
35 digested with *Hae*II to release the *lacZ'* fragment, treated
with Klenow enzyme to create blunt ends, and the *lacZ'*-
containing fragment ligated into pCGN565RB-H+X, which had
been digested with *Acc*I and *Sph*I and treated with Klenow

enzyme in such a orientation that the *lacZ'* promoter is proximal to the right border fragment; this construct, pCGN565RBα2x is positive for *lacZ'* expression when plated on an appropriate host and contains bp 13990-14273 of the

5 right border fragment (Barker et al., *Plant Mo. Biol.* (1983) 2:335-350) having deleted the *AccI-SphI* fragment (bp 13800-13990).

pCGN451

pCGN451 contains an ocs5'-ocs3' cassette, including

10 the T-DNA right border, cloned into a derivative of pUC8 (Vieira and Messing, *supra*). The modified vector is derived by digesting pUC8 with *HincII* and ligating in the presence of synthetic linker DNA, creating pCGN416, and then deleting the *EcoRI* site of pCGN416 by *EcoRI* digestion

15 followed by treatment with Klenow enzyme and self-ligation to create pCGN426.

The ocs5'-ocs3' cassette is created by a series of steps from DNA derived from the octopine Ti-plasmid pTiA6 (Currier and Nester, *supra*). To generate the 5'end, which

20 includes the T-DNA right border, an *EcoRI* fragment of pTiA6 (bp 13362-16202 (the numbering is by Barker, et al., *(Plant Mol. Bio* (1983) 2:335-350) for the closely related Ti plasmid pTi15955)) is removed from pVK232 (Knauf and Nester, *Plasmid* (1982) 8:45) by *EcoRI* digestion and cloned

25 into *EcoRI* digested pACYC184 (Chang and Cohen, *supra*) to generate pCGN15.

The 2.4kb *BamHI-EcoRI* fragment (bp 13774-16202) of pCGN15 is cloned into *EcoRI-BamHI* digested pBR322 (Bolivar, et al., *supra*) to yield pCGN429. The 412 bp *EcoRI-BamHI*

30 fragment (bp 13362-13772) of pCGN15 is cloned into *EcoRI-BamHI* digested pBR322 to yield pCGN407. The cut-down promoter fragment is obtained by digesting pCGN407 with *XmnI* (bp 13512), followed by resection with *Bal*31 exonuclease, ligation of synthetic *EcoRI* linkers, and

35 digestion with *BamHI*. Resulting fragments of approximately 130 bp are gel purified and cloned into M13mp9 (Vieira and Messing, *supra*) and sequenced. A clone, I-4, in which the *EcoRI* linker has been inserted at bp 1362 between the

transcription initiation point and the translation
initiation codon is identified by comparison with the
sequence of de Greve, et al., (J. Mol. Appl. Genet. (1982)
1:499-512). The EcoRI cleavage site is at position 13639,
5   downstream from the mRNA start site. The 141 bp EcoRI-
BamHI fragment of I-4, containing the cut-down promoter, is
cloned into EcoRI-BamHI digested pBR322 to create pCGN428.
The 141 bp EcoRI-BamHI promoter piece from pCGN428, and the
2.5 kb EcoRI-BamHI ocs5' piece from pCGN429 are cloned
10   together into EcoRI digested pUC19 (Vieira and Messing,
supra) to generate pCGN442, reconstructing the ocs upstream
region with a cut-down promoter section.

To generate the ocs3' end, the HindIII fragment of
pLB41 (D. Figurski, UC San Diego) containing the gentamycin
15   resistance gene is cloned into HindIII digested pACYC184
(Chang and Cohen, supra) to create pCGN413b. The 4.7 kb
BamHI fragment of pTiA6 (supra), containing the ocs3'
region, is cloned into BamHI digested pBR325 (F. Bolivar,
Gene (1978) 4:121-136) to create 33c-19. The SmaI site at
20   position 11207 (Barker, supra) of 33c-19 is converted to an
XhoI site using a synthetic XhoI linker, generating
pCCG401.2. The 3.8 kb BamHI-EcoRI fragment of pCGN401.2 is
cloned into BamHI-EcoRI digested pCGN413b to create
pCGN419.

25   The ocs5'-ocs3' cassette is generated by cloning the
2.64 kb EcoRI fragment of pCGN442, containing the 5'
region, into EcoRI digested pCGN419 to create pCNG446. The
3.1kb XhoI fragment of pCGN446, having the ocs5' region (bp
13639-15208) and ocs3' region (bp 11207-12823), is cloned
30   into the XhoI site of pCGN426 to create pCGN451.

Example 8

In this example, the preparation of a Bce-4 expression
cassette containing a plant desaturase is described.
35   The desaturase cDNA clone from pCGN2754 prepared as
described in Example 5, is modified by in vitro mutagenesis
to insert restriction sites immediately upstream of the ATG
start codon and downstream of the TGA stop codon. A

single-stranded template DNA is prepared for the
mutagenesis reaction from pCGN1894 (described in Example 6)
as described by Messing, (*Methods in Enzymol*. (1983)
101:20-79). Synthetic oligonucleotides are synthesized on

5   an Applied Biosystems 380A DNA synthesizer. The
oligonucleotides used are
    5'-CCATTTTTGATCTTCCTCGAGCCCGGGCTGCAGTTCTTCTTCTTCTTG-3'
(SEQ ID NO: 35) for the 5'mutagenesis and
    5'-GCTCGTTTTTTTTTTCTCTGCAGCCCGGGCTCGAGTCACAGCTTCACC -3'

10   (SEQ ID NO: 36) for the 3'-mutagenesis; both add *Pst*I, *Sma*I
and *Xho*I sites flanking the coding region. Both
oligonucleotides are 5'-phosphorylated (BRL 5'-Terminus
labelling kit) and used for mutagenesis with the pCGN1894
template by the procedure of Adelman et *al.* (*DNA* (1983)

15   *2*:183-193). Alternatively, the desired restriction sites
may be inserted by PCR, using the 3' oligo described above
(SEQ ID NO: 36) and another oligo,
5' ACTGACTGCAGCCCGGGCTCGAGGAAGATCAAAAATGGCTCTTC 3' (SEQ ID
NO: 37) for the 3' and 5' primers, respectively. The

20   template in this polymerase chain reaction is DNA from
pCGN1894. The *Xho*I fragment from the resulting clone can
be subcloned into the Bce4 expression cassette, pCGN1870
(described below) at the unique *Xho*I site. This
Bce4/desaturase expression cassette can then be inserted in

25   a suitable binary vector, transformed into *Agrobacterium
tumefaciens* strain EHA101 and used to transform plants as
provided in Example 10.

Bce-4 Expression Cassette

30   pCGN1870 is a Bce-4 expression cassette containing 5'
and 3' regulatory regions of the Bce-4 gene and may be
derived from the Bce-4 sequence found in pCGN1857, which
was deposited with the ATCC on March 9, 1990, and assigned
accession number 68251, or by methods known to one skilled

35   in the art from the sequence (SEQ ID NO: 27) provided in
Fig. 8. The Bce 4 gene may be isolated as follows:

    The *Cla*I fragment of pCGN1857, containing the Bce4
gene is ligated into *Cla*I digested Bluescript KS+

(Stratagene; La Jolla, CA), producing pCGN1864. Single stranded DNA is made from pCGN1864 and altered by *in vitro* mutagenesis using the oligonucleotides

BCE45P:

5   (5'GAGTAGTGAACTTCATGGATCCTCGAGGTCTTGAAAACCTAGA3') (SEQ ID NO: 38) and

BCE43P:

(5'CAATGTCTTGAGAGATCCCGGGATCCTTAACAACTAGGAAAAGG3') (SEQ ID NO: 39)

10   as described by Adelman *et al.* (*DNA* (1983) *2*:183-193). The oligonucleotide BSCP2 (5'GTAAGACACGACTTATCGCCACTG3') (SEQ ID NO: 40), complementary to a portion of Bluescript, is included in the reaction to improve the yield of double-stranded DNA molecules. The resulting plasmid, pCGN1866,

15   contains *Xho*I and *Bam*HI sites (from BCE45P) immediately 5' to the Bce4 start codon and *Bam*HI and *Sma*I sites (from BCE43P) immediately 3' to the Bce4 stop codon. The *Cla*I fragment of pCGN1866, containing the mutagenized sequences, is inserted into the *Cla*I site of pCGN2016

20   (described in Example 6), producing pCGN1866C. The *Cla*I fragment of pCGN1866C is used to replace the corresponding wild-type *Cla*I fragment of PCGN1867 (described below) to produce pCGN1868. Bce4 coding sequences are removed by digestion of pCGN1868 with *Bam*HI and recircularization of

25   the plasmid to produce pCGN1870. The Bce4 expression cassette, pCGN1870, contains 7.4 kb of 5' regulatory sequence and 1.9 kb of 3' regulatory sequence derived from the Bce4 genomic clone separated by the cloning sites, *Xho*I, *Bam*HI, and *Sma*I. Desaturase sequences in sense or

30   anti-sense orientation may be inserted into the cassette via the cloning sites and the resulting construct may be employed in a plant transformation technique.

pCGN1867

The *BamHI* and *SmaI* sites of pUC18 are removed by
BamHI-SmaI digestion and recircularizing of the plasmid,
without repair of the ends, to produce pCGN1862 The *PstI*
5   fragment of pCGN1857, containing the Bce4 gene, is inserted
into the *PstI* site of pCGN1862 to produce pCGN1867.

Example 9

In this example, the preparation of a napin 1-2
10   expression cassette containing a plant desaturase is
described.

Preparation of Desaturase Clone

The desaturase cDNA clone from pCGN2754 is prepared
15   and modified as described in Example 8. The *XhoI* fragment
from the resulting clone can be subcloned into the napin 1-
2 expression cassette, pCGN1808 (described below) at the
unique *XhoI* site. This napin 1-2/desaturase expression
cassette can then be inserted into a suitable binary
20   vector, transformed into *A. tumefaciens* strain EHA101 in a
like manner as described in Example 7.

Alternatively, the desaturase safflower clone may be
prepared such that restriction sites flank the translation
start and stop sites, as described in Example 8, with the
25   following modification. PCR was carried out according to
manufacturer's instructions except for the initial
annealing of the oligonucleotides to the template. The
reaction mix was heated to 90°C for 5 min, cooled to 37°C
over a one hour period, kept at 37°C for 20 min and then
30   subjected to standard PCR cycles. The PCR product was
digested with *PstI* and ligated to pUC8 (Vieira and Messing
(1982) *Gene 19*:2359-268) digested with *PstI* to produce
pCGN3220. The *NcoI/SacI* fragment of pCGN3220 containing
the pUC8 vector and the 5' and 3' sequences of the
35   safflower desaturase cDNA was gel purified and ligated to
the gel-purified cloned *NcoI/SacI* fragment from pCGN1894
(see Example 6). The resulting plasmid pCGN3222 contains
safflower desaturase cDNA sequences partially from the cDNA

clone and partially from the PCR. The regions obtained
from the PCR were confirmed by DNA sequencing as being
identical to the original cloned sequence.

5   *Expression Cassettes*
     Napin 1-2 pCGN1808 Expression Cassette
          An expression cassette utilizing 5' upstream sequences
     and 3' downstream sequences obtainable from *B. campestris*
     napin gene can be constructed as follows.
10   A 2.7 kb *Xho*I fragment of napin 1-2 (Fig. 10 and SEQ
     ID NO: 29) containing 5' upstream sequences is subcloned
     into pCGN789 (a pUC based vector the same as pUC119 with
     the normal polylinker replaced by the synthetic linker -
     5'GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3', SEQ ID
15   NO: 41, (which represented the polylinker *Eco*RI, *Sal*I,
     *Bgl*II, *Pst*I, *Xho*I, *Bam*HI, *Hind*III) and results in pCGN940.
     The majority of the napin coding region of pCGN940 was
     deleted by digestion with *Sal*I and religation to form
     pCGN1800. Single-stranded DNA from pCGN1800 was used in an
20   *in vitro* mutagenesis reaction (Adelman *et al.*, *DNA* (1983)
     2:183-193) using the synthetic oligonucleotide 5'
     GCTTGTTCGCCATGGATATCTTCTGTATGTTC 3', SEQ ID NO: 42. This
     oligonucleotide inserted an *Eco*RV and an *Nco*I restriction
     site at the junction of the promoter region and the ATG
25   start codon of the napin gene. An appropriate mutant was
     identified by hybridization to the oligonucleotide used for
     the mutagenesis and sequence analysis and named pCGN1801.
          A 1.7 kb promoter fragment was subcloned from pCGN1801
     by partial digestion with *Eco*RV and ligation to pCGN786 (a
30   pCGN566 chloramphenicol based vector with the synthetic
     linker described above in place of the normal polylinker)
     cut with *Eco*RI and blunted by filling in with DNA
     Polymerase I Klenow fragment to create pCGN1802.
          A 2.1 kb *Sal*I fragment of napin 1-2 (Fig. 10 and SEQ
35   ID NO: 29) containing 3' downstream sequences is subcloned
     into pCGN789 (described above) and results in pCGN941.
     pCGN941 is digested with *Xho*I and *Hind*III and the resulting
     approximately 1.6 kb of napin 3' sequences are inserted

into *XhoI-Hind*III digest d pCGN1802 to result in pCGN1803.
In order to remove a 326 nucleotide *Hind*III fragment
inserted opposite to its natural orientation, as a result
of the fact that there are 2 *Hind*III sites in pCGN1803, the

5   pCGN1803 is digested with *Hind*III and religated. Following
religation, a clone is selected which now contains only
1.25 kb of the original 1.6 napin 3' sequence. This clone,
pCGN1808 is the napin 1-2 expression cassette and contains
1.725 kb of napin promoter sequences and 1.265 kb of napin

10   3' sequence with the unique cloning sites *Sal*I, *Bgl*I, *Pst*I
and *Xho*I in between.

Napin 1-2 pCGN3223 Expression Cassette

Alternatively, pCGN1808 may be modified to contain

15   flanking restriction sites to allow movement of only the
expression sequences and not the antibiotic resistance
marker to binary vectors such as pCGN1557 (McBride and
Summerfelt, *supra*). Synthetic oligonucleotides containing
*Kpn*I, *Not*I and *Hind*III restriction sites are annealed and

20   ligated at the unique *Hind*III site of pCGN1808, such that
only one *Hind*III site is recovered. The resulting plasmid,
pCGN3200 contains unique *Hind*III, *Not*I and *Kpn*I restriction
sites at the 3'-end of the napin 3'-regulatory sequences as
confirmed by sequence analysis.

25   The majority of the napin expression cassette is
subcloned from pCGN3200 by digestion with *Hind*III and *Sac*I
and ligation to *Hind*III and *Sac*I digested pIC19R (Marsh, *et
al.* (1984) *Gene 32*:481-485) to make pCGN3212. The extreme
5'-sequences of the napin promoter region are reconstructed

30   by PCR using pCGN3200 as a template and two primers
flanking the *Sac*I site and the junction of the napin 5'-
promoter and the pUC backbone of pCGN3200 from the pCGN1808
construct. The forward primer contains *Cla*I, *Hind*III,
*Not*I, and *Kpn*I restiction sites as well as nucleotides 408-

35   423 of the napin 5'-sequence (from the *Eco*RV site) and the
reverse primer contains the complement to napin sequences
718-739 which include the unique *Sac*I site in the 5'-
promoter. The PCR was performed using a Perkin Elmer/Cetus

thermocycler according to manufacturer's specifications. The PCR fragment is subcloned as a blunt-ended fragment into pUC8 (Vieira and Messing (1982) *Gene* *19*:259-268) and digested with *HincII* to give pCGN3217. Sequence of

5   pCGN3217 across the napin insert verifies that no improper nucleotides were introduced by PCR. The napin 5-sequences in pCGN3217 are ligated to the remainder of the napin expression cassette by digestion with *ClaI* and *SacI* and ligation to pCGN3212 digested with *ClaI* and *SacI*. The

10 resulting expression cassette pCGN3221, is digested with *HindIII* and the napin expression sequences are gel purified away and ligated to pIC20H (Marsh, *supra*) digested with *HindIII*. The final expression cassette is pCGN3223, which contains in an ampicillin resistant background, essentially

15 identical 1.725 napin 5' and 1.265 3' regulatory sequences as found in pCGN1808. The regulatory regions are flanked with *HindIII*, *NotI* and *KpnI* restriction sites and unique *SalI*, *BglII*, *PstI*, and *XhoI* cloning sites are located between the 5' and 3' noncoding regions.

20   Desaturase sequences in sense or anti-sense orientation may be inserted into a napin expression cassette via the cloning sites. The resulting construct may be employed for plant transformation. For example, one of ordinary skill in the art could also use known

25 techniques of gene cloning, mutations, insertion and repair to allow cloning of a napin expression cassette into any suitable binary vector, such as pCGN1557 (described in Example 7) or other similar vectors.

30 *Desaturase Expression*
        The coding region of the safflower desaturase contained in pCGN3222 is cloned into the pCGN3223 napin cassette by digestion with *XhoI* and ligation to pCGN3223 digested with *XhoI* and *SalI*. The resulting plasmid,

35 pCGN3229 is digested with *Asp*718 and inserted in the binary vector pCGN1578 (McBride and Summerfelt (1990) *Plant Mol. Biol. 14*:269-276) at the unique *Asp*718 site. The resulting binary vector is pCGN3231 and contains the safflower

desaturase coding sequences flanked by the napin 5' and 3'
regulatory sequences as well as the plant selectable marker
construct, 35s/NPTII/tml.

The resulting binary vector, pCGN3231, is transformed
5   into *Agrobacterium* and utilized for plant transformation as
described in Example 10. For Northern analysis, total RNA
is isolated from day 21 and day 28 post-anthesis developing
seed from plants transformed with pCGN3231. Five samples
were analyzed at day 21 and two at day 28 post-anthesis.
10   RNA was isolated by the method of Hughes and Galau (*Plant
Mol. Biol. Reporter* (1988) *6*: 253-257). Northern blot
analysis was performed using a labeled 0.8 kb *Bgl*II
fragment of pCGN1894 as a probe. Prehybridization and
hybridization was at 42°C in 50% formamide, 10X Denhardt's
15   solution, 5X SSC, 0.1% SDS, 5mM EDTA and 100ug/ml denatured
salmon sperm DNA. Filters were washed at 55°C in 0.1 X
SSC, 0.1% SDS. Under these conditions, the probe does not
hybridize to the endogenous *Brassica* desaturase gene
sequences. mRNA complementary to the safflower desaturase
20   was detected in all the transgenic samples examined. More
mRNA was present at day 28 than at day 21 post-anthesis and
the highest level of RNA was seen in transgenic 3231-8.
The total safflower desaturase mRNA level was estimated to
be ~0.01% of the message at day 28 post-anthesis.
25   Western analysis (see below) gives a preliminary
indication of increased protein in one transformant, 3231-
8. However, the Western analysis is complicated by two
factors: 1. The presence of cross-reacting material at the
same molecular weight as expected for the safflower
30   desaturase. We believe this material is the endogenous
Brassica desaturase. 2. The analysis of levels of protein
expressed is also complicated by the normal developmental
increase in the expression of desaturase protein during
this time period. If the seeds examined are not at the
35   precise developmental stage as the control seeds,
quantitative differences in the amount of material seen may
be simply due to the normal increase in the *Brassica*

desaturase over this time period and not due to the
expression of the safflower desaturase.

Western Analysis

5   Soluble protein is extracted from developing seeds of
Brassica by homogenization with one volume (1ml/gram fresh
weight) of buffer containing 20mM potassium phosphate, pH
6.8. The homogenate is clarified by centrifugation at
12,000 x *g* for 10 minutes. A second centrifugation is
10   performed if necessary to provide a non-particulate
supernatant.
        Protein concentration of the extract is measured by
the micromethod of Bradford (*Anal. Biochem.* (1976) 72:248-
254). Proteins (20-60μg) are separated by denaturing
15   electrophoresis by the method of Laemmli (*supra*), and are
transferred to nitrocellulose membrane by the method of
Towbin *et al.* (*Proc. Nat. Acad Sci.* (1979) 76:4350-4354).
        The nitrocellulose membrane is blocked by incubation
at room temperature for 15 minutes or at 4°C overnight in
20   Tris-buffered saline with Tween 20 (Polyoxyethylenesorbitan
monolaurate) and "TTBS-milk", (TTBS = 20mM Tris-HCl, 500mM
NaCl, 0.1% Tween 20 (v/v), pH 7.5; "TTBS-milk" = TTBS and
3% skim milk powder). The volume of liquid in all
incubations with the nitrocellulose membrane is sufficient
25   to cover the membrane completely. The membrane is then
incubated for an additional 5 minutes in TTBS.
        The nitrocellulose membrane is incubated for at least
one hour with shaking at room temperature with rabbit anti-
stearoyl-ACP desaturase antiserum that was diluted 5,000-
30   or 10,000-fold in "TTBS-milk". The rabbit anti-desaturase
antiserum was commercially prepared from desaturase protein
(purified as described in Example 1) by Berkeley Antibody
Co. (Richmond, CA). The membrane is washed twice by
shaking with TTBS for 5 minutes and then with deionized H_2O
35   for 30 seconds.
        The nitrocellulose membrane is incubated for at least
45 minutes at room temperature in a solution of "TTBS-milk"
in which anti-rabbit IgG-alkaline phosphatase conjugate

(Promega, Madison, WI) is diluted 7,500-fold. The membrane
is washed twice in TTBS followed by deionized H_2O, as
described above.

The nitrocellulose membrane is equilibrated in buffer
5 containing 100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5,
by shaking for 5 minutes. The color reaction is initiated
by placing the nitrocellulose membrane into 50ml of the
same buffer to which has been added 15mg p-nitroblue
tetrazolium chloride and 7.5mg 5-bromo- 4 chloro- 3-indolyl
10 phosphate toluidine salt (BioRad Labs; Richmond, CA). The
color reaction is stopped by rinsing the nitrocellulose
membrane with deionized H_2O and drying it between filter
papers.

Oil analysis of developing seeds indicated no
15 significant change in oil composition of the transformed
plants with respect to the control plants. This result is
consistant with the low levels of safflower mRNA observed
in transgenic plants as compared to levels of endogenous
Brassica desaturase (Example 12).

20

Example 10

In this example, an *Agrobacterium*-mediated plant
transformation is described. *Brassica napus* is
exemplified. The method is also useful for transformation
25 of other *Brassica* species including *Brassica campestris*.

Plant Material and Transformation

Seeds of *Brassica napus* cv. Delta are soaked in 95%
ethanol for 2 min, surface sterilized in a 1.0% solution of
30 sodium hypochlorite containing a drop of Tween 20 for 45
min., and rinsed three times in sterile, distilled water.
Seeds are then plated in Magenta boxes with 1/10th
concentration of Murashige minimal organics medium (Gibco)
supplemented with pyrodoxine (50 µg/l), nicotinic acid (50
35 µg/l), glycine (200 µg/l), and 0.6% Phytagar (Gibco) pH
5.8. Seeds are germinated in a culture room at 22°C in a
16 h photoperiod with cool fluorescent and red light of

intensity approximately 65 µEinsteins per square meter per second ($\mu Em^{-2}s^{-1}$).

Hypocotyls are excised from 7 day old seedlings, cut into pieces approximately 4 mm in length, and plated on

5   feeder plates (Horsch et al. 1985). Feeder plates are prepared one day before use by plating 1.0 ml of a tobacco suspension culture onto a petri plate (100x25 mm) containing about 30 ml MS salt base (Carolina Biological) 100 mg/l inositol, 1.3 mg/l thiamine-HCl, 200 mg KH_2PO_4

10   with 3% sucrose, 2,4-D (1.0 mg/l), 0.6% Phytagar, and pH adjusted to 5.8 prior to autoclaving (MS0/1/0 medium). A sterile filter paper disc (Whatman 3 mm) is placed on top of the feeder layer prior to use. Tobacco suspension cultures are subcultured weekly by transfer of 10 ml of

15   culture into 100 ml fresh MS medium as described for the feeder plates with 2,4-D (0.2 mg/l), Kinetin (0.1 mg/l). All hypocotyl explants are preincubated on feeder plates for 24 h. at 22°C in continuous light of intensity 30 $\mu Em^{-2}s^{-1}$ to 65 $\mu EM^{-2}s^{-1}$.

20   Single colonies of *A. tumefaciens* strain EHA101 containing a binary plasmid are transferred to 5 ml MG/L broth and grown overnight at 30°C. Per liter, MG/L broth contains 5g mannitol, 1 g L-glutamic acid or 1.15 g sodium glutamate, 0.25 g kH_2PO_4, 0.10 g NaCL, 0.10 g $MGSO_4 \cdot 7H_2O$, 1

25   mg biotin, 5 g tryptone, and 2.5 g yeast extract, and the broth is adjusted to pH 7.0. Hypocotyl explants are immersed in 7-12 ml MG/L broth with bacteria diluted to 1×10^8 bacteria/ml and after 10-20 min. are placed onto feeder plates. After 48 h of co-incubation with

30   *Agrobacterium*, the hypocotyl explants are transferred to B5 0/1/0 callus induction medium which contains filter sterilized carbenicillin (500 mg/l, added after autoclaving) and kanamycin sulfate (Boehringer Mannheim) at concentrations of 25 mg/l.

35   After 3-7 days in culture at 65 $\mu Em^{-2}s^{-1}$ to 75 $\mu Em^{-2}s^{-1}$ continuous light, callus tissue is visible on the cut surface and the hypocotyl explants are transferred to shoot induction medium, B5BZ (B5 salts and vitamins supplemented

with 3 mg/l benzylaminopurine, 1 mg/l zeatin, 1% sucrose,
0.6% Phytagar and pH adjusted to 5.8). This medium also
contains carbenicillin (500 mg/l) and kanamycin sulfate (25
mg/l). Hypocotyl explants are subcultured onto fresh shoot
5   induction medium every two weeks.

Shoots regenerate from the hypocotyl calli after one
to three months. Green shoots at least 1 cm tall are
excised from the calli and placed on medium containing B5
salts and vitamins, 1% sucrose, carbenicillin (300 mg/l),
10 kanamycin sulfate (50 mg/l) and 0.6% Phytagar) and placed
in a culture room with conditions as described for seed
germination. After 2-4 weeks shoots which remain green are
cut at the base and transferred to Magenta boxes containing
root induction medium (B5 salts and vitamins, 1% sucrose, 2
15 mg/l indolebutyric acid, 50 mg/l kanamycin sulfate and 0.6%
Phytagar). Green rooted shoots are tested for NPT II
activity.

Example 11

20   In this example, a DNA-bombardment plant transformation is
described. Peanut transformation is exemplified.

DNA sequences of interest may be introduced as
expression cassettes, comprising at least a promoter
region, a gene of interest, and a termination region, into
25 a plant genome via particle bombardment as described in
European Patent Application 332 855 and in co-pending
application USSN 07/225,332, filed July 27, 1988.

Briefly, tungsten or gold particles of a size ranging
from 0.5μM-3μM are coated with DNA of an expression
30 cassette. This DNA may be in the form of an aqueous
mixture or a dry DNA/particle precipitate.

Tissue used as the target for bombardment may be from
cotyledonary explants, shoot meristems, immature leaflets,
or anthers.
35   The bombardment of the tissue with the DNA-coated
particles is carried out using a Biolistics™ particle gun
(Dupont; Wilmington, DE). The particles are placed in the
barrel at variable distances ranging from 1cm-14cm from the

barrel mouth. The tissue to be bombarded is placed beneath the stopping plate; testing is performed on the tissue at distances up to 20 cm. At the moment of discharge, the tissue is protected by a nylon net or a combination of
5 nylon nets with mesh ranging from 10μM to 300μM.

Following bombardment, plants may be regenerated following the method of Atreya, et al., (Plant Science Letters (1984) 34:379-383). Briefly, embryo axis tissue or cotyledon segments are placed on MS medium (Murashige and
10 Skoog, Physio. Plant. (1962) 15:473) (MS plus 2.0 mg.1 6-benzyladenine (BA) for the cotyledon segments) and incubated in the dark for 1 week at 25 ± 2°C and are subsequently transferred to continuous cool white fluorescent light (6.8 W/m^2). On the 10th day of culture,
15 the plantlets are transferred to pots containing sterile soil, are kept in the shade for 3-5 days are and finally moved to greenhouse.

The putative transgenic shoots are rooted. Integration of exogenous DNA into the plant genome may be
20 confirmed by various methods known to those skilled in the art.

Example 12

This example describes methods to obtain desaturase
25 cDNA clones from other plant species using the DNA from the C. tinctorius Δ-9 desaturase clone as the probe.

Isolation of RNA for Northern Analysis
Poly(A)+ RNA is isolated from C. tinctorius embryos
30 collected at 14-17 days post-anthesis and Simmondsia chinensis embryos as described in Example 5.

Total RNA is isolated from days 17-18 days post-anthesis Brassica campestris embryos by an RNA minipreparation technique (Scherer and Knauf, Plant Mol.
35 Biol. (1987) 9:127-134). Total RNA is isolated from R. communis immature endosperm of about 14-21 days post-anthesis by a method described by Halling, et al. (Nucl. Acids Res. (1985) 13:8019-8033). Total RNA is isolated

64

from 10 g each of young leaves from *B. campestris*, *B. napus*, and *C. tinctorius*, by extraction of each sample in 5 ml/g tissue of 4 M guanidine thiocyanate buffer as described by Colbert *et al.* (*Proc. Nat. Acac. Sci.* (1983)

5   *80*:2248-2252). Total RNA is also isolated from immature ·embryos of *Cuphea hookeriana* by extraction as above in 10 ml/g tissue.

Total RNA is isolated from immature embryos of California bay (*Umbellularia californica*) by an adaptation

10 of the method of Lagrimini *et al.* (*Proc. Nat. Acad. Sci.* (1987) *84*:7542-7546). Following homogenization in grinding buffer (2.5 ml/g tissue) as described, RNA is precipitated from the aqueous phase by addition of 1/10 volume 3 M sodium acetate and 2 volumes ethanol, followed by freezing

15 at -80°C for 30 minutes and centrifugation at 13,000 x g for 20 minutes. The pellets are washed with 80% ethanol and centrifugation is repeated as above. The pellets are resuspended in water, two volumes of 4 M LiCl are added, and the samples are placed at -20°C overnight. Samples are

20 centrifuged as above and the pellets washed with 80% ethanol. Ethanol precipitation is repeated as above.

Total RNA is further purified from *B. campestris*, *B. napus*, and *C. tinctorius* leaves, and from *C. tinctorius*, *B. campestris*, California bay, and jojoba, and from *R.*

25 *communis* immature endosperm, by removing polysaccharides on a 0.25 g Sigma Cell 50 cellulose column. The RNA is loaded onto the column in 1 ml of loading buffer (20 mM Tris-HCl pH 7.5, 0.5M NaCl, 1mM EDTA, 0.1% SDS), eluted with loading buffer, and collected in 500 µl fractions. Ethanol is

30 added to the samples to precipitate the RNA. The samples are centrifuged, and the pellets resuspended in sterile distilled water, pooled, and again precipitated in ethanol. The sample is centrifuged, and the resulting RNA is subjected to oligo(dT)-cellulose chromatography to enrich

35 for poly(A)+ RNA as described by Maniatis *et al.* (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1982)). Poly(A)+ RNA is also

purified from total *Cuphea hookeriana* RNA by oligo(dT)-
cellulose chromatography.

   *Northern Analysis Using C. tinctorius Desaturase
Clone:* 2.5 µg of poly(A)+ RNA from each of the above

5   described poly(A)+ samples from immature embryos of jojoba,
Cuphea hookeriana, California bay, *Brassica campestris*, and
C. tinctorius, from immature endosperm of *R. communis*, and
from leaves of *C. tinctorius*, *B. campestris*, and *B. napus*
are electrophoresed on formaldehyde/agarose gels (Fourney

10 *et al., Focus* (1988) *10*:5-7) and transferred to a Hybond-C
extra (Amersham, Arlington Heights, IL) filter according to
manufacturer's specifications. The filter is prehybridized
for four hours and hybridized overnight at 42°C in a roller
bottle containing 10 ml of hybridization buffer (1 M NaCl,

15 1% SDS, 50% formamide, 0.1 mg/ml denatured salmon sperm
DNA) in a Hybridization Incubator, model 1040-00-1 (Robbins
Scientific Corporation, Sunnyvale, CA). The probe used in
the hybridization is a gel-isolated *Bgl*II fragment of the
Δ-9 desaturase clone that is labeled with ^{32}P-dCTP using a

20 BRL (Gaithersburg, MD) nick-translation kit, following
manufacturer's instructions. The blot is washed three
times for 20 minutes each in 2X SSC, 0.5% SDS at 55°C. The
blot is exposed at -80°C, with a Dupont Cronex intensifying
screen, to X-ray film for four days.

25   The autoradiograph shows that the *C. tinctorius*
desaturase gene is expressed in both immature embryos and
leaves of *C. tinctorius*, although the level of expression
is considerably higher in embryos than in leaves. The
autoradiograph also shows hybridization of the *C.*

30 *tinctorius* desaturase clone to mRNA bands of a similar size
in immature embryos from jojoba and California bay, and
immature endosperm from *R. communis*. Hybridization is also
detectable in RNA from *B. campestris* embryos upon longer
exposure of the filter to X-ray film.

35   *R. communis cDNA Library Construction:* A plant seed
cDNA library may be constructed from poly(A)+ RNA isolated
from *R. communis* immature endosperm as described above.
The plasmid cloning vector pCGN1703, and cloning method are

as described in Example 5. The *R. communis* endosperm cDNA
bank contains approximately $2x10^6$ clones with an average
cDNA insert size of approximately 1000 base pairs.

The *R. communis* immature endosperm cDNA bank is moved
5   into the cloning vector lambda gt22 (Stratagene Cloning
Systems) by digestion of total cDNA with *NotI* and ligation
to lambda gt22 DNA digested with *NotI*. The resulting phage
are packaged using a commercially available kit and titered
using *E. coli* strain LE392 (Stratagene Cloning Systems, La
10   Jolla, CA). The titer of the resulting library was
approximately 1.5×10^7 pfu/ml.

R. communis cDNA *Library Screen*: The library is
plated on *E. coli* strain LE392 at a density of
approximately 25,000 pfu/150mm NZY plate to provide
15   approximately 50,000 plaques for screening. Phage are
lifted in duplicate on to NEN (Boston, MA.) Colony/Plaque
Screen filters as described in Example 5. Following
prehybridization at 42°C in 25 ml of hybridization buffer
(1 M NaCl, 1% SDS, 50% formamide, 0.1 mg/ml denatured
20   salmon sperm DNA) filters are hybridized overnight with a
gel-purified 520 base pair *BglII* fragment of the *C.
tinctorius* desaturase clone (Figure 7A) that is
radiolabeled with ^{32}P-dCTP using a BRL (Gaithersburg, MD)
Nick Translation System. Filters are washed three times
25   for 20 minutes each in 2X SSC, 0.5% SDS at 55°C in a
shaking water bath. Filters are exposed to X-ray film
overnight at -80°C with a Dupont Cronex intensifying
screen.

Clones are detected by hybridization on duplicate
30   filters with the *C. tinctorius* desaturase cDNA fragment and
plaque purified. During plaque purification, it was
observed that larger plaques were obtained when *E. coli*
strain Y1090 (Young, R.A. and Davis, R.W., *Proc. Natl.
Acad. Sci. USA* (1983) *80*:1194) was used as the host
35   strain. This strain was thus used in subsequent plaque
purification steps. Phage DNA is prepared from the
purified clones as described by Grossberger (*NAR* (1987)
15:6737) with the following modification. The proteinase K

treatment is replaced by the addition of 10% SDS and a 10
minute incubation at room temperature. Recovered phage DNA
is digested with *EcoRI*, religated at low concentration, and
transformed into *E. coli* DH5α(BRL; Gaithersburg, MD) cells

5   to recover plasmids containing cDNA inserts in pCGN1703.
Minipreparation DNA (Maniatis *et al.*, *supra*) is prepared
from the clones and DNA sequence is determined as described
above. Partial nucleotide sequence of the cDNA insert of a
R. communis desaturase clone pCGN3230 is presented in

10 Figure 3A and SEQ ID NO: 14. The complete nucleotide
sequence of this clone is presented in Fig. 3B and as SEQ
ID NO: 15.

Northern Analysis Using R. communis Desaturase Clone:
Total RNA for Northern analysis is isolated from tobacco

15 leaves by the method of Ursin *et al.*(*Plant Cell* (1989)
1:727-736), petunia and tomato leaves by the method of
Ecker and Davis (*Proc.Nat.Acad.Sci.* (1987) *84*:5202-5206),
and corn leaves by the method of Turpen and Griffith
(*Biotechniques* (1986) *4*:11-15). Total RNA samples from

20 tobacco, corn, and tomato leaves are enriched for poly(A)+
RNA by oligo(dT)-cellulose chromatography as described by
Maniatis *et al.* (*supra*).

Poly(A)+ RNA samples from tomato leaves (4 µg) and
corn and tobacco leaves (1 µg each), and total RNA from

25 petunia leaves (25 µg) are electrophoresed on a
formaldehyde/agarose gel as described by Shewmaker *et al.*
(*Virology* (1985) *140*:281-288). Also electrophoresed on
this gel are poly(A)+ RNA samples isolated from *B.
campestris* day 17-19 embryos and *B. campestris* leaves (2 µg

30 each), immature embryos from *C. tinctorius*, bay, and jojoba
(1 µg each), and *R. communis* endosperm (1 µg). The
isolation of these poly(A)+ RNA samples is described above
for the Northern analysis using *C. tinctorius* desaturase
cDNA as probe. The RNA is transferred to a nitrocellulose

35 filter as described by Shewmaker *et al.* (*supra*) and
prehybridized and hybridized at 42°C in 50% formamide, 10X
Denhardt's solution (described in Maniatis *et al.* (supra)),
5X SSC, 0.1% SDS, 5 mM EDTA, 100 ug/ml denatured salmon

sperm DNA, and 10% dextran sulfate (in hybridization buffer only). The probe for hybridization is the ^{32}P-labeled (BRL Nick Translation Kit) 1.7 kb SalI insert of pCGN3230 that has been gel-purified from minipreparation DNA. The filter

5   is washed following hybridization for 30 minutes in 2X SSC, 0.1% SDS at 42°C and at 50°C twice for 15 minutes each. The filter is exposed to X-ray film overnight at -80°C with a Dupont Cronex intensifying screen.

The autoradiograph shows hybridization of the *R.*
10 *communis* desaturase clone to mRNA bands of a similar size in immature embryos from *B. campestris*, California bay, and *C. tinctorius*, and also in corn leaves and *R. communis* endosperm.

B. campestris Embryo cDNA Library Construction: Total
15 RNA is isolated from 5 g of *B. campestris* cv. R500 embryos obtained from seeds harvested at days 17-19 post-anthesis. RNA is extracted in 25 mls of 4 M guanidine thiocyanate buffer as described by Colbert et al.(PNAS (1983) *80*:2248- 2252). Polysaccharides are removed from the RNA sample by
20 resuspending the pellet in 6 ml of 1X TE (10 mM Tris/1 mM EDTA pH 8), adding potassium acetate to a concentration of 0.05M, and adding one half volume of ethanol. The sample is placed on ice for 60 minutes and centrifuged for 10 minutes at 3000 x g. RNA is precipitated from the supernatant by
25 adding sodium acetate to a concentration of 0.3 M followed by the addition of two volumes of ethanol. RNA is recovered from the sample by centrifugation at 12,000 x g for 10 minutes and yield calculated by UV spectrophotometry. Two mg of the total RNA is further purified by removing
30 polysaccharides on a 0.25 g Sigma Cell 50 cellulose column, as described above, and is also enriched for poly(A)+ RNA by oligo(dT)-cellulose chromatography as described above.

A *B. campestris* day 17-19 post anthesis embryo cDNA library is constructed in plasmid vector pCGN1703 as
35 described in Example 5, using 5 ug of the above described poly(A)+ RNA. The library, which consists of approximately 1.5 x 10^5 transformants, is amplified by plating and scraping colonies, and is stored as frozen *E. coli* cells in

10% DMSO at -80° C.   DNA is isolated from a portion of the
amplified library by scaling up the alkaline lysis
technique of Birnboim and Doly (*Nucleic Acids Res.* (1979)
7:1513), and purified by CsCl centrifugation.   Library DNA
5   is digested with *EcoRI* and is cloned into *EcoRI*-digested
bacteriophage lambda gt10 (Stratagene; La Jolla, CA) DNA.
The DNA is packaged using Gigapack II Gold *in vitro*
packaging extracts (Stratagene; La Jolla, CA) according to
manufacturer's specifications.   The titer of the phage
10   stock, determined by dilution plating of phage in *E. coli*
C600 hfl- cells (Huynh, *et al., DNA Cloning. Volume 1.*
Eds. Gover, D.M. (1985) IRL Press Limited: Oxford,
England, pp. 56,110), is 6 x 10^6 pfu per ml.

     B. campestris cDNA Library Screen: The library is
15   plated on *E. coli* strain C600 hfl- at a density of
approximately 30,000 pfu/150mm NZY plate to provide
approximately 120,000 plaques for screening.   Phage are
lifted in duplicate on to NEN (Boston, MA.) Colony/Plaque
Screen filters as described in Example 5.   Filters are
20   prehybridized and hybridized with the ^{32}P-labeled fragment
of pCGN3230 as described above for the Northern
hybridization.   Filters are washed for 30 minutes in 2X
SSC, 0.1% SDS at 50°C and at 55°C twice for 15 minutes
each.   Filters are exposed to X-ray film overnight at -80°C
25   with a Dupont Cronex intensifying screen.

     Clones are detected by hybridization on duplicate
filters to the *R. communis* desaturase cDNA fragment and
plaque purified.   During plaque purification, the probe
used was a gel-purified 1.4 kb SstI fragment of pCGN3230
30   which lacks the poly(A)+ tail.   As described above, phage
DNA is isolated from purified lambda clones, digested with
EcoRI, ligated, and transformed to *E. coli* DH5α cells.
Minipreparation DNA is prepared and partial DNA sequence
determined as described above.   Partial DNA sequences of
35   two clones, pCGN3235 and pCGN3236, are presented in Figure
4A (SEQ ID NO: 17) and 4B (SEQ ID NO: 18), respectively.
Initial DNA sequence analysis of the 3' regions of these
clones indicates that pCGN3236 and pCGN3235 are cDNA

clones from the same gen . pCGN3236 is a shorter clone
than pCGN3235, which appears to contain the entire coding
region of the *B. campestris* desaturase gene. The complete
nucleotide sequence of pCGN3235 is presented in Figure 4C
5   and SEQ ID NO: 19.

Desaturase Gene Analysis: Southern and Northern
analyses of Brassica species are conducted to determine the
number of genes which encode the Brassica desaturase clone,
pCGN3235 in *B. campestris*, *B. oleracea*, and *B. napus*, and
10   the timing of expression of the gene in *B. campestris*
developing seeds. DNA is isolated from leaves of each of
the above-named *Brassica* species by the method of Bernatzky
and Tanksley (*Theor. Appl. Genet.* (1986) *72*:314-321). DNA
from each of the species is digested with restriction
15   endonucleases EcoRI and XbaI (10 ug/digest),
electrophoresed in a 0.7% agarose gel, and transferred to a
nitrocellulose filter (Maniatis *et al.*, *supra*). The filter
is prehybridized and hybridized at 42°C (as described above
for Northern analysis using *R. communis* desaturase clone)
20   with a ^{32}P-labeled (nick translation) gel-isolated
HindIII/PvuII fragment of pCGN3235 (Fig. 7C). The filter
is washed following overnight hybridization, for 30 minutes
at 55°C in 1X SSC, 0.1% SDS, followed by two 15 minute
washed at 55°C in 0.1X SSC, 0.1% SDS.

25   The autoradiograph indicates that the *Brassica*
desaturase is encoded by a small gene family consisting of
about two genes in *B. campestris* and *B. oleracea*, and about
four genes in *B. napus*.

The timing of expression of the desaturase gene during
30   seed development is determined by Northern analysis. RNA
is isolated from immature seeds of *B. campestris* cv. R500
collected at 11, 13, 15, 17, 19, 21, 25, 30, 35, and 40
days post-anthesis. Total RNA is isolated as described by
Scherer and Knauf (*Plant Mol. Biol.* (1987) *9*:127-134).
35   Twenty five micrograms of RNA from each time point are
electrophoresed through a formaldehyde-containing 1.5%
agarose gel as described by Shewmaker, et *al.* (*supra*) and
blotted to nitrocellulose (Thomas, *Proc. Nat. Acad. Sci.*

(1980) 77:5201-5205). The blot is pre-hybridized and
hybridized at 42°C with the ^{32}P-labeled HindIII/PvuII
fragment of pCGN3235 as described above. The filter is
washed following overnight hybridization, for 30 minutes at
5   55°C in 1X SSC, 0.1% SDS, followed by two 15 minute washed
at 55°C in 0.1X SSC, 0.1% SDS.

The autoradiograph indicates that the desaturase gene
is expressed in *B. campestris* developing seeds beginning at
about day 19 and through about day 30, with maximal
10   expression at day 25. By a similar Northern analysis, the
level of desaturase mRNA in developing *Brassica napus* seeds
(day 21) was estimated to be approximately 1% of the total
mRNA.

Isolation of Other Desaturase Gene Sequences: cDNA
15   libraries may be constructed as described above and genomic
libraries can be constructed from DNA from various sources
using commercially available vectors and published DNA
isolation, fractionation, and cloning procedures. For
example, a *B. campestris* genomic library can be constructed
20   using DNA isolated according to Scofield and Crouch
(J.Biol.Chem. (1987) 262:12202-12208) that is digested with
BamHI and fractionated on sucrose gradients (Maniatis *et
al., supra*), and cloned into the lambda phage vector
LambdaGem-11 (Promega; Madison,WI) using cloning procedures
25   of Maniatis *et al.* (*supra*).

cDNA and genomic libraries can be screened for
desaturase cDNA and genomic clones, respectively, using
published hybridization techniques. Screening techniques
are described above for screening libraries with DNA
30   fragments. Libraries may also be screened with synthetic
oligonucleotides, for example using methods described by
Berent *et al.* (*BioTechniques* (1985) 3:208-220). Probes for
the library screening can be prepared by PCR, or from the
sequences of the desaturase clones provided herein.
35   Oligonucleotides prepared from the desaturase sequences may
be used, as well as longer DNA fragments, up to the entire
desaturase clone.

For example, jojoba polyadenylated RNA is used to construct a cDNA library in the cloning vector λZAPII/*EcoRI* (Stratagene, San Diego, CA). RNA is isolated from jojoba embryos collected at 80-90 days post-anthesis by isolating

5   polyribosomes using a method initially described by Jackson and Larkins (*Plant Physiol.* (1976) *57*:5-10) and modified by Goldberg *et al.* (*Developmental Biol.* (1981) *83*:201-217). Polysaccharide contaminants in the polyribosomal RNA preparation are removed by running the RNA over a cellulose

10 column (Sigma-cell 50) in high salt buffer (0.5M NaCl, 20mM Tris pH 7.5, 1mM EDTA, 0.1% SDS). The contaminant binds to the column and the RNA is collected in the eluant. The eluant fractions are pooled and the RNA is ethanol precipitated. The precipitated total RNA is then

15 resuspended in a smaller volume and applied to an oligo d(T) cellulose column to isolate the polyadenylated RNA.

The library is constructed using protocols, DNA and bacterial strains as supplied by the manufacturer. Clones are packaged using Gigapack Gold packaging extracts

20 (Stratagene), also according to manufacturer's recommendations. The cDNA library constructed in this manner contins approximately 1 x 10^6 clones with an average cDNA insert size of approximately 400 base pairs.

The jojoba library is plated on *E. coli* XL1-Blue

25 (Stratagene) at a density of approximately 5000pfu/150mm plate to provide approximately 60,000 plaques for screening. Phage are lifted onto duplicate nylon membrane filters as described previously. Filters are prehybridized at 42°C in a hybridization buffer containing 40% formamide,

30 10X Denhardt's solution, 5X SSC, 0.1% SDS, 50mM EDTA, and 100μg/ml denatured salmon sperm DNA. Hybridization is at 42°C in the same buffer with added nick translated (BRL Nick Translation System) 520 bp *Bgl*II fragment of the *C. tinctorius* desaturase clone described previously. Filters

35 are washed at 50°C in 2X SSC and exposed to X-ray film overnight.

Desaturase clones are detected by hybridization on duplicate filters with the *C. tinctorius* cDNA fragment and

plaque-purified. Positive clones are recovered as plasmids
in *E. coli* following manufacturer's directions and
materials for *in vivo* excision. Partial, preliminary DNA
sequence of a clone, 3-1, is determined and the

5   corresponding amino acid sequence is translated in three
frames. In this manner, homology to the *C. tinctorius*
desaturase cDNA clone is detected in one reading frame.
The preliminary DNA sequence of this jojoba desaturase cDNA
fragment is shown in F:gure 5 (SEQ ID NO: 43). Also shown

10   is the corresponding translated amino acid sequence in the
reading frame having *C. tinctorius* desaturase homology.
The jojoba cDNA fragment is approximately 75% homologous at
the DNA level and approximately 79% homologous at the amino
acid level compared to sequence of the *C. tinctorius*

15   desaturase in this region.

Example 13

Antisense constructs are described which allow for
transcription of a reverse copy of the *B. campestris*

20   desaturase cDNA clone in the 5' to 3' orientation of
transcription.

Preferential Expression of Antisense Constructs in Embryos

In order to reduce the transcription of a desaturase

25   gene in embryos of *B. napus* or *B. campestris*, constructs
may be prepared which allow for production of antisense
copies of the desaturase cDNA preferentially in the
embryos. Promoter sequences which are desirable to obtain
this pattern of expression include, but are not limited to,

30   the ACP, Bce4, and napin 1-2 expression cassettes described
in Examples 7, 8, and 9, respectively. It also may be
desirable to control the expression of reverse copies of
the desaturase cDNA under two different promoters in the
same transformed plant to provide for a broader timing of

35   expression of the antisense desaturase DNA. For example,
expression from the ACP promoter may begin and end earlier
than expression from the napin promoter. Thus, expressing
the reverse desaturase from both promoters may result in

the production of the antisense strand of DNA over a longer
period of embryo development.

An example of expression of an antisense desaturase
gene preferentially in the embryos is provided below.

5   Similar constructs containing the same or a different
fragment of the desaturase gene and any of the promoters
described above, as well as other promoter regions which
may be useful, may also be prepared using gene cloning,
insertion, mutation and repair techniques well known to

10 those of ordinary skill in the art.

A.   Antisense Desaturase Expression from the ACP Promoter
     Construction of pCGN3239 is as follows:
     pCGN3235 (Example 12) is digested with PvuII and

15 HindIII and the HindIII sticky ends are filled in with
     Klenow in the presence of 200 µM dNTPs. The 1.2 kb
     PvuII/HindIII fragment containing the desaturase coding
     sequence is gel purified and ligated in the antisense
     orientation into EcoRV-digested pCGN1977 (ACP expression

20 cassette; described in Example 7) to create pCGN3238. The
     4.2 kb XbaI/Asp718 fragment of pCGN3238 containing the
     antisense desaturase in the ACP cassette is transferred
     into XbaI/Asp718-digested pCGN1557 (binary transformation
     vector; described in Example 7) to create pCGN3239.

25 B.   Antisense Desaturase Expression From The Napin
     Promoter
     Construction of pCGN3240 is as follows: pCGN3235 is
     digested with PvuII and HindIII, the sticky ends are
     blunted, and the resulting fragment is inserted in an anti-

30 sense orientation into pCGN3223 which has been digested
     with SalI and blunted with Klenow enzyme. The resulting
     plasmid, pCGN3240 will express an anti-sense desaturase RNA
     from the napin promoter cassette.

     C.   Antisense Desaturase Expression From a Dual Promoter
35 Cassette
     Construction of pCGN3242 is as follows: An Asp718
     fragment of pCGN3240 containing the napin 5' and 3' regions
     surrounding the desaturase sequences is inserted into the

*Asp*718 site of pCGN3239 (a binary vector containing an ACP
promoter, antisense desaturase construct) to create
pCGN3242.

5   *Constitutive Transcription*
     A.   <u>Binary Vector Construction</u>
     1.   Construction of pCGP291.
            The *Kpn*I, *Bam*HI, and *Xba*I sites of binary vector
     pCGN1559 (McBride and Summerfelt, *Pl.Mol.Biol.* (1990) *14*:
10   269-276) are removed by *Asp*718/*Xba*I digestion followed by
     blunting the ends and recircularization to produce pCGP67.
     The 1.84 kb *Pst*I/*Hind*III fragment of pCGN986 containing the
     35S promoter-tml3' cassette is inserted into *Pst*I/*Hind*III
     digested pCGP67 to produce pCGP291.
15   2.   Construction of pCGN986.
            The 35S promoter-tml3' expression cassette, pCGN986,
     contains a cauliflower mosaic virus 35S (CaMV35) promoter
     and a T-DNA tml 3'-region with multiple restriction sites
     between them.   pCGN986 is derived from another cassette,
20   pCGN206, containing a CaMV35S promoter and a different 3'
     region, the CaMV region VI 3'-end.   The CaMV 35S promoter
     is cloned as an *Alu*I fragment (bp 7144-7734) (Gardner *et.
     al., Nucl.Acids Res.* (1981) *9*:2871-2888) into the *Hinc*II
     site of M13mp7 (Messing, *et. al., Nucl.Acids Res.* (1981)
25   *9*:309-321) to create C614.   An *Eco*RI digest of C614
     produced the *Eco*RI fragment from C614 containing the 35S
     promoter which is cloned into the *Eco*RI site of pUC8
     (Vieira and Messing, *Gene* (1982) *19*:259) to produce
     pCGN147.
30   pCGN148a containing a promoter region, selectable
     marker (KAN with 2 ATG's) and 3' region, is prepared by
     digesting pCGN528 with *Bgl*II and inserting the *Bam*HI-*Bgl*II
     promoter fragment from pCGN147.   This fragment is cloned
     into the *Bgl*II site of pCGN528 so that the *Bgl*II site is
35   proximal to the kanamycin gene of pCGN528.
            The shuttle vector used for this construct, pCGN528,
     is made as follows: pCGN525 is made by digesting a plasmid
     containing Tn5 which harbors a kanamycin gene (Jorgenson

et. al., Mol. Gen. Genet. (1979) 177:65) with HindIII-BamHI
and inserting the HindIII-BamHI fragment containing the
kanamycin gene into the HindIII-BamHI sites in the
tetracycline gene of pACYC184 (Chang and Cohen, J.
5   Bacteriol. (1978) 134:1141-1156). pCGN526 was made by
inserting the BamHI fragment 19 of pTiA6 (Thomashow et.
al., Cell (1980) 19:729-739), modified with XhoI linkers
inserted into the SmaI site, into the BamHI site of
pCGN525. pCGN528 is obtained by deleting the small XhoI
10   fragment from pCGN526 by digesting with XhoI and
religating.

     pCGN149a is made by cloning the BamHI-kanamycin gene
fragment from pMB9KanXXI into the BamHI site of pCGN148a.
pMB9KanXXI is a pUC4K variant (Vieira and Messing, Gene
15   (1982) 19:259-268) which has the XhoI site missing, but
contains a functional kanamycin gene from Tn903 to allow
for efficient selection in Agrobacterium.

     pCGN149a is digested with HindIII and BamHI and
ligated to pUC8 digested with HindIII and BamHI to produce
20   pCGN169. This removes the Tn903 kanamycin marker. pCGN565
and pCGN169 are both digested with HindIII and PstI and
ligated to form pCGN203, a plasmid containing the CaMV 35S
promoter and part of the 5'-end of the Tn5 kanamycin gene
(up to the PstI site, Jorgenson et. al., (1979), supra). A
25   3'-regulatory region is added to pCGN203 from pCGN204, an
EcoRI fragment of CaMV (bp 408-6105) containing the region
VI 3' cloned into pUC18 (Yanisch-Perron, et al., Gene
(1985) 33:103-119) by digestion with HindIII and PstI and
ligation. The resulting cassette, pCGN206, is the basis
30   for the construction of pCGN986.

     The pTiA6 T-DNA tml 3'-sequences are subcloned from
the Bam19 T-DNA fragment (Thomashow et al., (1980) supra)
as a BamHI-EcoRI fragment (nucleotides 9062 to 12,823,
numbering as in Barker et al., Plant Mol. Biol. (1982)
35   2:335-350) and combined with the pACYC184 (Chang and Cohen
(1978), supra) origin of replication as an EcoRI-HindIII
fragment and a gentamycin resistance marker (from plasmid

pLB41), obtained from D. Figurski) as a BamHI-HindIII
fragment to produce pCGN417.

The unique SmaI site of pCGN417 (nucleotide 11,207 of
the Bam19 fragment) is changed to a SacI site using linkers

5   and the BamHI-SacI fragment is subcloned into pCGN565 to
give pCGN971. The BamHI site of pCGN971 is changed to an
EcoRI site using linkers. The resulting EcoRI-SacI
fragment containing the tml 3' regulatory sequences is
joined to pCGN206 by digestion with EcoRI and SacI to give

10   pCGN975. The small part of the Tn5 kanamycin resistance
gene is deleted from the 3'-end of the CaMV 35S promoter by
digestion with SalI and BglII, blunting the ends and
ligation with SalI linkers. The final expression cassette
pCGN986 contains the CaMV 35S promoter followed by two SalI

15   sites, an XbaI site, BamHI, SmaI, KpnI and the tml 3'
region (nucleotides 11207-9023 of the T-DNA).

B.   Insertion of Desaturase Sequence

The 1.6 kb XbaI fragment from pCGN3235 containing the
desaturase cDNA is inserted in the antisense orientation

20   into the XbaI site of pCGP291 to produce pCGN3234.

Plant Transformation

The binary vectors containing the expression cassette
and the desaturase gene are transformed into *Agrobacterium*

25   *tumefaciens* strain EHA101 (Hood, et al., J. Bacteriol.
(1986) 168:1291-1301) as per the method of Holsters, et
al., Mol. Gen. Genet. (1978) 163:181-187. Transformed B.
napus and/or Brassica campestris plants are obtained as
described in Example 10.

30

Analysis of Transgenic Plants

A.   Analysis of pCGN3242 Transformed Brassica campestris
cv. Tobin Plants

Due to the self-incompatibility of Brassica campestris

35   cv. Tobin, individual transgenic plants are pollinated
using non-transformed Tobin pollen. Because of this, the
T2 seeds of a transgenic plant containing the antisense
desaturase at one locus would be expected to segregate in a

1:1 ratio of transformed to non-transformed seed. The oil
composition of ten individual seeds collected at 26 days
post-anthesis from several pCGN3242-transformed plants and
one non-transformed control was analyzed by gas

5   chromatography according to the method of Browse, *et al.,*
Anal. Biochem. (1986) *152*:141-145. One transformant, 3242-
T-1, exhibits an oil composition that differed distinctly
from controls on preliminary analysis. The control Tobin
seeds contained an average of 1.8% 18:0 (range 1.5% - 2.0%)

10 and 52.9% 18:1 (range 48.2% - 57.1%). T2 seeds of 3242-T-1
segregated into two distinct classes. Five seeds contained
levels of 18:0 ranging from 1.3% to 1.9% and levels of 18:1
ranging from 42.2% to 58.3%. The other five seeds
contained from 22.9% to 26.3% 18:0 and from 19.9% to 26.1%

15 18:1.

    B.   Analysis of pCGN3234 Transformed Plants

          Some abnormalities have been observed in some
    transgenic *Brassica napus* cv. Delta and Bingo and *Brassica*
    campestris cv. Tobin plants containing pCGN3234. These

20 effects could be due to the constitutive expression of
    antisense desaturase RNA from the 35S promoter or could be
    due to the transformation/tissue culture regime the plants
    have been subjected to.

          The above results demonstrate the ability to obtain

25 plant Δ-9 desaturases, isolate DNA sequences which encode
    desaturase activity and manipulate them. In this way, the
    production of transcription cassettes, including expression
    cassettes can be produced which allow for production,
    including specially differentiated cell production of the

30 desired product. A purified *C. tinctorius* desaturase is
    provided and used to obtain nucleic acid sequences of *C.*
    tinctorius desaturase. Other plant desaturase sequences
    are provided such as *R. cummunis, B. campestris,* and *S.*
    chinensis. These sequences as well as desaturase sequences

35 obtained from them may be used to obtain additional
    desaturease, and so on. And, as described in the
    application modification of oil composition may be
    achieved.

All publications and patent applications mentioned in
this specification are indicative of the level of skill of
those skilled in the art to which this invention pertains.
5   All publications and patent applications are herein
incorporated by reference to the same extent as if each
individual publication or patent application was
specifically and individually indicated to be incorporated
by reference.
10   Although the foregoing invention has been described in
some detail by way of illustration and example for purposes
of clarity of understanding, it will be obvious that
certain changes and modifications may be practiced within
the scope of the appended claim.
15

What is claimed is:

1.   A recombinant DNA construct comprising a sequence encoding at least a portion of a plant desaturase, said desaturase when mature having activity toward an
5   unsaturated fatty acid substrate.

2.   The construct of Claim 1 encoding a biologically active plant desaturase.

3.   The construct of Claim 1 wherein said sequence encodes a precursor desaturase.

10   4.   The construct of Claim 1 wherein said sequence encodes a mature desaturase.

5.   The construct of Claim 1 wherein said sequence encodes a transit peptide.

6.   The construct of Claim 1 comprising a cDNA
15   sequence.

7.   The construct of Claim 1 wherein said sequence is joined to a second nucleic acid sequence which is not naturally joined to said first sequence.

8.   The construct of Claim 1 comprising, in the 5' to
20   3' direction of transcription, a transcriptional regulatory region functional in a host cell and said sequence.

9.   The construct of Claim 8 further comprising, a translational regulatory region immediately 5' to said sequence and a transcriptional/translational termination
25   regulatory region 3' to said sequence, wherein said regulatory regions are functional in said host cell.

10.   The construct of Claim 8 wherein said sequence is a sense sequence.

11.   The construct of Claim 8 wherein said sequence is
30   an anti-sense sequence.

12.   The construct of Claim 8 wherein said host cell is a plant cell.

13.   The construct of Claim 12 wherein said transcriptional initiation region is obtained from a gene
35   preferentially expressed in plant seed tissue during lipid accumulation.

14.   The construct of Claim 13 wherein said transcriptional initiation region is selected from the

regulatory region 5' upstream to a structural gene of the group consisting of any one of Bce4, seed ACP Bcg 4-4 and napin 1-2.

15.   The construct of Claim 9 wherein said transcriptional termination region is a plant desaturase termination region.

16.   The construct of Claim 1 wherein said plant desaturase is a Δ-9 desaturase.

17.   The construct of Claim 1 wherein said sequence is obtainable from any one of *C. tinctorius*, *R. communinis* and *B. campestris*.

18.   A method of modifying fatty acid composition in a plant host cell from a given percentage of fatty acid saturation to a different percentage of fatty acid saturation comprising

growing a host plant cell having integrated into its genome a recombinant DNA sequence encoding a plant desaturase under the control of regulatory elements functional in said plant cell during lipid accumulation under conditions which will promote the activity of said regulatory elements.

19.   The method of Claim 18 wherein the overexpression of plant desaturase is obtained.

20.   The method of Claim 18 wherein the decrease of endogenous plant desaturase is obtained.

21.   The method of Claim 18 wherein said regulatory elements function preferentially in plant seed.

22.   The method of Claim 20 wherein the percentage of long chain unsaturated fatty acids is increased.

23.   A plant cell having a modified level of saturated fatty acids produced according to the method of any one of Claims 18-22.

24.   The plant cell of Claim 23 wherein said cell is a *Brassica* plant cell.

25.   The plant cell of Claim 23 wherein said cell is *in vivo*.

26.   The plant cell of Claim 23 wherein said cell is an oilseed embryo plant cell.

27.   A plant seed having a modified level of saturated
fatty acids as compared to a seed of said plant having a
native level of saturated fatty acids produced according to
a method comprising
5   growing a plant, having integrated into the genome of
embryo cells a recombinant DNA sequence encoding a plant
desaturase under the control of regulatory elements
functional in seed during lipid accumulation, to produce
seed under conditions which will promote the activity of
10 said regulatory elements, and
           harvesting said seed.
           28.   The seed of Claim 27 wherein said plant is
Brassica napus.
           29.   The seed of claim 27 wherein said seed is an
15 oilseed.
           30.   The seed of Claim 27 wherein said plant
desaturase is a Δ-9 desaturase.
           31.   A plant seed oil of a plant having an endogenous
level of saturated fatty acids comprising a plant seed oil
20 having a modified level of saturated fatty acids.
           32.   The oil of Claim 31 comprising a *Brassica napus*
oil.
           33.   A plant seed oil separated from an seed produced
according to any one of Claims 27-30.
25   34.   A host cell comprising a plant desaturase
encoding sequence of any one of Claims 1-17.
           35.   The cell of Claim 34 wherein said cell is a plant
cell.
           36.   The cell of Claim 35 wherein said plant cell is
30 *in vivo*.
           37.   The cell of Claim 35 wherein said plant cell is a
Brassica plant cell.
           38.   A transgenic host cell comprising an expressed
plant desaturase.
35   39.   The cell of Claim 38 wherein said host cell is a
plant cell.
           40.   The cell of Claim 38 wherein said plant
desaturase is a Δ-9 desaturase.

41. A method of producing a plant desaturase in a host cell or progeny thereof comprising

growing a host cell or progeny thereof comprising a construct of any one of Claims 1-10 and 12-17 under

5   conditions which will permit the production of said plant desaturase.

42. The method of Claim 41 wherein said host cell is a plant cell and said construct is integrated into the genome of said plant cell.

10   43. The method of Claim 42 wherein said plant cell is *in vivo*.

44. A host cell comprising a plant desaturase produced according to Claim 41.

45. The cell of Claim 45 wherein said host cell is a

15   plant host cell and said construct is integrated into the genome of said plant cell.

F1: ASTLGSSTPKVDNAKKPFQPPREVHVQVTH$^{S}_{X}$MPPQKIEIFKSIEG$^{W}_{R}$AEQNILV$^{H}_{F}$LKPVEKCWQ

F2: DFLPDPA$^{S}_{T}$EGFDEQVKELRARAKEIPDDYFVVLVGDMITEEALPTYQTMLNTLDGV

F3: DETGASLITPWAVWT

F4: DLLHTYLYLSGRV

F5: DMRQIQKTIQYLI

F6: TENSPYLGFIYTSFQER

F7: DV$^{K}_{F}$LAQI$^{C}_{Q}$GTIASDEKRHETAYTKIVEKLFEIDPDGTVLAFADMRKKI$^{S}_{T}$MPAHLMY

F8: DNLF

F9: dvFlAV$^{A}_{I}$QRL$^{G}_{I}$VYTAK

F10: DYADILEFLVGRWK

F11: VADLTGLSGEGRKA$^{Q}_{G}$DYVCGLPPRIRRLEERAQGRAKEGPVVPFSWIFDRQVKL

FIGURE 1

HindIII
|

```
1   GCTCACTTGTGTGGTGGAGGAGAGAAAAAACAGAACTCACAAAAAGCTTTGCGACTGCCAAGAACAACAACA     69
                                              42
```
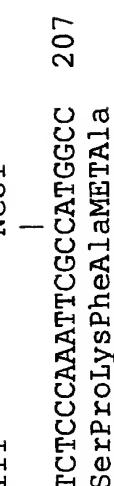
```
70  ACAACAAGATCAAGAGAGAGAAGAGAAGAAGATCAAAAATGGCTCTCGAATCACTCCAGTGACCTTGCAA      138
                                         METAlaLeuArgIleThrProValThrLeuGln
```

                                    EcoRV   BglII   NcoI
                                      | | |
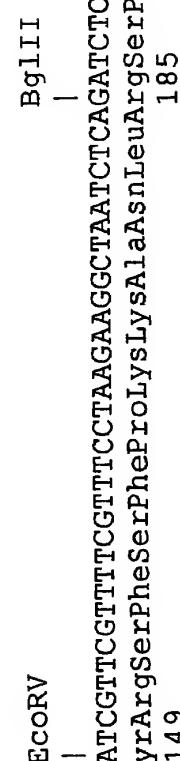
```
139 TCGGAGAGATATCGTTCGTTTCCTAAGAAGGCTAATCTCCCAAATTCGCCATGGCC                   207
    SerGluArgTyrArgSerPheSerPheProLysLysAlaAsnLeuArgSerProLysPheAlaMETAla
                    149                              185                 201
```
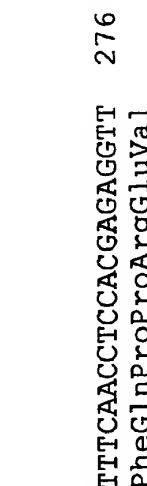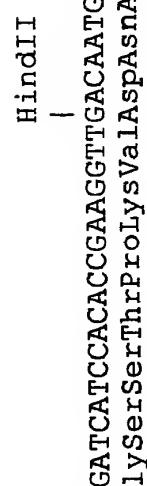
                                    HindII
                                      |

```
208 TCCACCCTCGGATCATCCACACCGAAGGTTGACAATGCCAAGAAGCCTTTTCAACCTCCACGAGAGGTT      276
    SerThrLeuGlySerSerThrProLysValAspAsnAlaLysLysProPheGlnProProArgGluVal
                              238
```
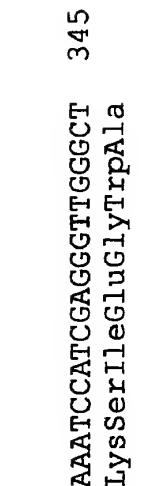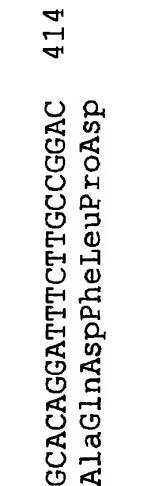
```
277 CATGTTCAGGTGACGCACTCCATGCCACCACAGAAGATAGAGATTTCAAATCCATCGAGGGTTGGGCT      345
    HisValGlnValThrHisSerMETProProGlnLysIleGluIlePheLysSerIleGluGlyTrpAla
```

```
346 GAGCAGAACATATTGGTTCACCTAAAGCCAGTGGAGAAATGTTGGCAAGCACAGGATTCTTGCCGGAC      414
    GluGlnAsnIleLeuValHisLeuLysProValGluLysCysTrpGlnAlaGlnAspPheLeuProProAsp
```

FIGURE 2
Page 1 of 4

415 CCTGCATCTGAAGGATTTGATGAACAAGTCAAGGAACTAAGGGCAAGAGCAAAGGAGGAGATTCCTGATGAT 483
ProAlaSerGluGlyPheAspGluGlnValLysGluLeuArgAlaArgAlaLysGluGluIleProAspAsp

484 TACTTTGTTGTTTGGTTGGAGATATGATTACAGAGAGGAAGCCCTACCTACCAAACAATGCTTAAT 552
TyrPheValValLeuValGlyAspMETIleThrGluGluAlaLeuProThrTyrGlnThrMETLeuAsn

553 ACCCTAGATGGTGTACGTGATGAGACTGGGGCTAGCCTTACGCGCCTTGGGCTGTGTCTGGACTAGGGCTTGG 621
ThrLeuAspGlyValArgAspGluThrGlyAlaSerLeuThrProTrpAlaValTrpThrArgAlaTrp

PvuII
——

622 ACAGCTGAAGAGAACAGGCATGGCGATCTTCTCCACACCTATCTCTACCTTTCTGGGCGGGTAGACATG 690
ThrAlaGluGluAsnArgHisGlyAspLeuLeuHisThrTyrLeuTyrLeuSerGlyArgValAspMET
626   684

AccI
——

691 AGGCAGATACAGAAGACAATTCAGTATCCTCATTGGGTCAGGAATGGATCCTCGTACCGAAAACAGCCCC 759
ArgGlnIleGlnLysThrIleGlnTyrLeuIleGlySerGlyMETAspProArgThrGluAsnSerPro
736

BamHI
——

760 TACCTTGGGTTCATCTACACATCGTTTCAAGAGCGTGCCACATTGTTTCTCACGGAAACACCGCCAGG 828
TyrLeuGlyPheIleTyrThrSerPheGlnGluArgAlaThrLeuPheValSerHisGlyAsnThrAlaArg

FIGURE 2
Page 2 of 4

```
SphI
 |
829 CATGCAAAGGATCATGGGGACGTGAAACTGGCGCAAATTTGTGGTACAATCGCGTCTGACGAAAAGCGT  897
    HisAlaLysAspHisGlyAspValLysLeuAlaGlnIleCysGlyThrIleAlaSerAspGluLysArg
                                       833

                                                        ClaI
                                                         |
898 CACGAGACCGGCTTATACAAAGATAGTCGAAAAGCTATTCGAGATCGATGGCACCGTTCTTGCT  966
    HisGluThrAlaTyrThrLysIleValGluLysLeuPheGluIleAspProAspGlyThrValLeuAla
                                       942

                             BglII
                              |
967 TTTGCCACATGATGAGGAAAAAGATCTCGATGCCCGCCACACTTGATGTACGATGGGCGTGATGACAAC  1035
    PheAlaAspMETMETArgLysLysIleSerMETProAlaHisLeuMETTyrAspGlyArgAspAspAsn
                              990

                                          AccI
                                           |
1036 CTCTTCGAACATTTCTCGGCGGTTGCCCAAAGACTCGGCGTCTACACCGCCAAAGACTACGCCGACATA  1104
     LeuPheGluHisPheSerAlaValAlaGlnArgLeuGlyValTyrThrAlaLysAspTyrAlaAspIle
                                          1077

1105 CTGGAATTTCTGGTCGGGCGGTGGAAAGTGGCGGGATTTGACCGGCCTATCTGGTGAAGGGCGTAAAGCG  1173
     LeuGluPheLeuValGlyArgTrpLysValAlaAlaAspLeuThrGlyLeuSerGlyGluGlyArgLysAla
```

FIGURE 2
Page 3 of 4

```
                                                    SacI
                                                     |
1174  CAAGATTATGTTTGCGGGTTGCCACCAAGAATCAGAAGGCTGGAGGAGAGAGCTCAAGGGGCGAGCAAAG 1242
      GlnAspTyrValCysGlyLeuProProArgIleArgArgLeuGluArgAlaGlnGlyArgAlaLys
                                                                       1228

                          PvuII
                           |
1243  GAAGGACCTGTTGTTCCATTCAGCTGGATTTTCGATAGACAGGTGAAGCTGTGAAGAAAAAAAAACGA 1311
      GluGlyProValValProPheSerTrpIlePheAspArgGlnValLysLeu
                                                        1266

1312  GCAGTGAGTTCGGTTTCTCGTTGGCTTATTGGGTAGAGGTTAAAACCTATTTTAGATGTCTGTTTCGTGT 1380

1381  AATGTGGTTTTTTTTCTTCTAAATCTTGAATCTGGTATTGTGTCGTTGAGTTCGCGTGTGTAAACTTG 1449

1450  TGTGGCTGTGGACATATTATAGAACTCGTTATGCCAATTTTGATGACGGTGGTTATCGTCTCCCCTGGT 1518

1519  GTTTTTTATTGTTT 1533
```

FIGURE 2
Page 4 of 4

1 AAAAGAAAAAGGTAAGAAAAAAAAAACAATGGCTCTCAAGCTCTCAATCCTTCCTTTCTCAAACCCAAAAGT 69
                            METAlaLeuLysLeuSerGlnThrGlnLysL
METAlaLeuLysLeuAsnProPheLeuSerGlnThrGlnLysL

BglII

70 TACCTTCTTTCGCTCTTCCACCAATGGCCAGTACCCCTAAGTTCTACATGGCCTCTACCCTCA 138
   euProSerPheAlaLeuProProMETAlaSerThrArgSerProLysPheTyrMETAlaSerThrLeuL

139 AGTCTGGTTCTAAGGAAGTTGAGAATCTCAAGAAGCCTTTCATGCCTCCTCGGGAGGTACATGTTCAGG 207
    ysSerGlySerLysGluValGluAsnLeuLysLysProPheMETProProArgGluValHisValGlnV

208 TTACCCATTCTATTGCCA 225
    alThrHisSerIleAla

FIGURE 3A

```
                                                                    56
AAAAGAAAAA GGTAAGAAAA AAAACA ATG GCT CTC AAG CTC AAT CCT TTC CTT TCT
                             MET Ala Leu Lys Leu Asn Pro Phe Leu Ser

                                                                    110
CAA ACC CAA AAG TTA CCT TCT GCT CTT CCA CCA ATG GCC AGT ACC AGA TCT
Gln Thr Gln Lys Leu Pro Ser Phe Ala Leu Pro Pro MET Ala Ser Thr Arg Ser

                                                                    164
CCT AAG TTC TAC ATG GCC TCT ACC CTC AAG TCT GGT TCT AAG GAA GTT GAG AAT
Pro Lys Phe Tyr MET Ala Ser Thr Leu Lys Ser Gly Ser Lys Glu Val Glu Asn

                                                                    218
CTC AAG AAG CCT TTC ATG CCT CGG GAG GTA CAT GTT CAG CAT GTT ACC CAT TCT
Leu Lys Lys Pro Phe MET Pro Arg Glu Val His Val Gln His Val Thr His Ser

                                                                    272
ATG CCA CCC CAA AAG ATT GAG ATC TTT AAA TCC CTA GAC AAT TGG GCT GAG GAG
MET Pro Pro Gln Lys Ile Glu Ile Phe Lys Ser Leu Asp Asn Trp Ala Glu Glu

                                                                    326
AAC ATT CTG GTT CAT CTG AAG CCA GTT CCG CAA TGT TGG AAA CCG CAG GAT TTT
Asn Ile Leu Val His Leu Lys Pro Val Pro Gln Cys Trp Lys Pro Gln Asp Phe

                                                                    380
TTG CCA GAT CCC GCC TCT GAT GGA TTT GAT GAG CAA GTC AGG GAG
Leu Pro Asp Pro Ala Ser Asp Gly Phe Asp Glu Gln Val Arg Glu

                                                                    434
AGA GCA AAG GAG ATT CCT GAT TAT GAT CCT GGA GAC ATG ATA
Arg Ala Lys Glu Ile Pro Asp Tyr Asp Pro Gly Asp MET Ile

                                                                    488
ACG GAA GAA GCC CTT CCC ACT TAT CAA ACA ATG CTG AAT ACC TTG GAT GGA GTT
Thr Glu Glu Ala Leu Pro Thr Tyr Gln Thr MET Leu Asn Thr Leu Asp Gly Val
```

FIGURE 3B
Page 1 of 3

```
542   CGG GAT GAA ACA GGT GCA AGT CCT ACT TCT TGG GCA ATT TGG ACA AGG GCA TGG
      Arg Asp Glu Thr Gly Ala Ser Pro Thr Ser Trp Ala Ile Trp Thr Arg Ala Trp

596   ACT GCG GAA AAT AGA CAT GGT GAC CTC AAT CTC TAC AAG TAT CTA TCT
      Thr Ala Glu Asn Arg His Gly Asp Leu Asn Leu Tyr Lys Tyr Leu Ser

650   GGA CGA GTG GAC ATG CAA ATT GAG ACA TAT CAA TTG ATT GGT TCA
      Gly Arg Val Asp MET Gln Ile Glu Thr Tyr Gln Leu Ile Gly Ser

704   GGA ATG GAT CCA CGG ACA GAA AAC AGT CCA TAC ATC TAT ACA TCA
      Gly MET Asp Pro Arg Thr Glu Asn Ser Pro Tyr Ile Tyr Thr Ser

758   TTC CAG GAA AGG GCA ACC TTC ATT TCT CAT GGG AAC ACT GCC CGA AAA
      Phe Gln Glu Arg Ala Thr Phe Ile Ser His Gly Asn Thr Ala Arg Lys

812   GAG CAT GGA GAC ATA AAG TTG GCT CAA ATA TGT ACA ATT GCT GCA GAT GAG
      Glu His Gly Asp Ile Lys Leu Ala Gln Ile Cys Thr Ile Ala Ala Asp Glu

866   AAG CGC CAT GAG ACA GCC TAC ACA AAG ATA GTG GAA CTC TTT GAG ATT GAT
      Lys Arg His Glu Thr Ala Tyr Thr Lys Ile Val Glu Leu Phe Glu Ile Asp

920   CCT GAT GGA ACT GTT TTG GCT TTT GAT ATG ATG AGA AAG AAA ATT TCT ATG
      Pro Asp Gly Thr Val Leu Ala Phe Asp MET MET Arg Lys Lys Ile Ser MET

974   CCT GCA CAC TTG ATG TAT GGC CGA GAT GAT AAT CTT GAC CAC TTT TCA
      Pro Ala His Leu MET Tyr Gly Arg Asp Asp Asn Leu Asp His Phe Ser
```

FIGURE 3B
Page 2 of 3

```
                                                                      1028
GCT GTT GCG CAG CGT CTT GGA GTC TAC ACA GCA AAG GAT TAT GCA GAT ATA TTG
Ala Val Ala Gln Arg Leu Gly Val Tyr Thr Ala Lys Asp Tyr Ala Asp Ile Leu

                                                                      1082
GAG TTC TTG GTG GGC AGA TGG GAT AAA GTG GAT AAG GTG GAT CTT TCA GCT GAG
Glu Phe Leu Val Gly Arg Trp Asp Lys Val Asp Lys Val Asp Leu Ser Ala Glu

                                                                      1136
GGA CAA AAG GCT CAG GAC TAT GTT TGT CGG TTA CCT CCA AGA ATT AGA AGG CTG
Gly Gln Lys Ala Gln Asp Tyr Val Cys Arg Leu Pro Pro Arg Ile Arg Arg Leu

                                                                      1190
GAA GAG AGA GCT CAA GGA AGG GCA AAG GAA GCA AAG GAA GCA CCC ACC ATG CCT TTC AGC TGG
Glu Glu Arg Ala Gln Gly Arg Ala Lys Glu Ala Lys Glu Ala Pro Thr MET Pro Phe Ser Trp

                                                                      1254
ATT TTC GAT AGG CAA GTG AAG CTG TAGGTGGCTA AAGTGCAGGA CGAAACCGAA ATGGTTAGTT
Ile Phe Asp Arg Gln Val Lys Leu

                                                                      1324
TCACTCTTTT TCATGCCCAT CCCTGCAGAA TCAGAAGTAG AGGTAGAATT TTGTAGTTGC TTTTTATTA

                                                                      1394
CAAGTCCAGT TTAGTTAAG GTCTGTGGAA GGGAGTTAGT TGAGGAGTGA ATTTAGTAAG TTGTAGATAC

                                                                      1464
AGTTGTTTCT TGTGTTGTCA TGAGTATGCT GATAGAGAGC AGCTGTAGTT TTGTTGTTGT GTTCTTTTAT

                                                                      1534
ATGGTCTCTT GTATGAGTTT CTTTTCTTTC CTTTTCTTCT TTCCTTTCCT CTCTCTCTCT CTCTCTCTCT

                                                                      1604
CTCTTTTTCT CTTATCCCAA GTGTCTCAAG TATAATAAGC AAACGATCCA TGTGGCAATT TTGATGATGG

                                                                      1668
TGATCAGTCT CACAACTTGA TCTTTTGTCT TCTATTGGAA ACACAGCCTG CTTGTTTGAA AAAA
```

FIGURE 3B
Page 3 of 3

pCGN3235

1 TGAGAGATAGTGTGAGAGCATTAGCCTTAGAGAGAGAGAGAGAGCTTGTGTCTGAAAGAATCCACAA 69

HindIII
|

70 ATGGCATTGAAGCTTAACCCTTGGCATCTCAGCCTTACAACTTCCCT 117
   METAlaLeuLysLeuAsnProLeuAlaSerGlnProTyrAsnPhePro

FIGURE 4A

pCGN3236

PstI
|

1 ACTTCATGGGCTATTTGGACAAGAGAGCTTGGACTGCAGAAGAGAGAACCGACACGGTGATCTTCTCAATAAG 69
ThrSerTrpAlaIleTrpThrArgAlaTrpThrArgAlaGluGluAsnArgHisGlyAspLeuLeuAsnLys

70 TATCTTTACTTGTCTGGACGTGTTGACATGAGGCAGATTGAAAAGACCATTCAGTACTTGATTGGTTCT 138
TyrLeuTyrLeuSerGlyArgValAspMETArgGlnIleGluLysThrIleGlnTyrLeuIleGlySer

BamHI
|

139 GGAATGGATCCTAGAACAGAGAACAATCCTTACCTCGG 176
GlyMETAspProArgThrGluAsnAsnProTyrLeuAla

FIGURE 4B

pCGN3235

```
TGAGAGATAG TGTGAGAGCA TTAGCCTTAG AGAGAGAGAG AGAGAGCTTG TGTCTGAAAG AATCCACAA

ATG GCA TTG AAG CTT AAC CCT GCA TCT CAG CCT TAC AAC TTC CCT TCC TCG
MET Ala Leu Lys Leu Asn Pro Ala Ser Gln Pro Tyr Asn Phe Pro Ser Ser

GCT CGT CCG ATC TCT ACT TTC AGA TCT CCC AAG TTC CTC TGC GCT TCT
Ala Arg Pro Ile Ser Thr Phe Arg Ser Pro Lys Phe Leu Cys Ala Ser

TCT CCC GCT CTC AGC TCC AAG GAG AGT TTG GAG AAG CCA TTC ACA
Ser Pro Ala Leu Ser Ser Lys Glu Ser Leu Glu Lys Pro Phe Thr

CCA CCT AAG GAA GTG CAC GTT CAA GTC CTG CAT TCC CCA CCC CAG AAG ATC
Pro Pro Lys Glu Val His Val Gln Val Leu His Ser Pro Pro Gln Lys Ile

GAG ATC TTC AAA TCC ATG GAA GAC TGG GCC GAG CAG AAC CTT CTA ACT CAG CTC
Glu Ile Phe Lys Ser MET Glu Asp Trp Ala Glu Gln Asn Leu Leu Thr Gln Leu

AAA GAC GTG GAG AAG TCG TGG CCC CAG CCC GAC TTC TTA CCC GAC GCA TCC
Lys Asp Val Glu Lys Ser Trp Pro Gln Pro Asp Phe Leu Pro Asp Ala Ser

GAT GGG TTC GAA GAT CAG GTT GAT GTT AGA GAG CTA CAG GTT GCA AGG GAG CCT
Asp Gly Phe Glu Asp Gln Val Asp Val Arg Glu Leu Gln Val Ala Arg Glu Pro

GAT GAT TAC TTC GTT CTG GTG GGA GAC ATG ATC ACG GAA GAG GCG CTT CCG
Asp Asp Tyr Phe Val Leu Val Gly Asp MET Ile Thr Glu Glu Ala Leu Pro
```

FIGURE 4C
Page 1 of 3

```
ACC TAT CAA ACC ATG TTG AAC ACT TTG GAT GGA GTG AGG GAT GAA ACT GGC GCT
Thr Tyr Gln Thr MET Leu Asn Thr Leu Asp Gly Val Arg Asp Glu Thr Gly Ala

AGC CCC ACT TCA TGG GCT ATT TGG ACA AGA GCT GCA GAG GAA AAC CGA
Ser Pro Thr Trp Ala Ile Trp Thr Arg Ala Trp Ala Glu Glu Asn Arg

CAC GGT GAT CTT CTC AAT AAG TAT CTT TCT GGA CGT GTT GAC ATG AGG
His Gly Asp Leu Leu Asn Lys Tyr Leu Ser Gly Arg Val Asp MET Arg

CAG ATT GAA AAG ACC ATT CAG TAC TTG ATT GGT TCT GGA ATG CCT AGA ACA
Gln Ile Glu Lys Thr Ile Gln Tyr Leu Ile Gly Ser GGA MET Asp Pro Arg Thr

GAG AAC AAT CCT TAC CTC GGC TTC ATC TAC TCA TTC CAA GAA AGA GCC ACC
Glu Asn Asn Pro Tyr Leu Gly Phe Ile Tyr Ser Phe Gln Glu Arg Ala Thr

TTC ATC TCT CAC GGA AAC ACA GCT CGC CAA GCC AAA GAG CAC GGA GAC CTC AAG
Phe Ile Ser His Gly Asn Thr Ala Arg Gln Ala Lys Glu His Gly Asp Leu Lys

CTA GCC CAA ATC TGC GGC ACA ATA GCT GCA GAC GAG CGT CAT GAG ACA GCT
Leu Ala Gln Ile Cys Gly Thr Ile Ala Ala Asp Glu Arg His Glu Thr Ala

TAC ACC AAG ATA GTT GAG AAG CTC TTT GAG ATT GAT GGT CCT GAT ACT GTG ATG
Tyr Thr Lys Ile Val Glu Lys Leu Phe Glu Ile Asp Gly Pro Asp Thr Val MET

GCG TTT GCA GAC ATG AGG ATG AAA ATC TCG ATG CCT GCT CAC TTG ATG TAC
Ala Phe Ala Asp MET Arg MET Lys Ile Ser MET Pro Ala His Leu MET Tyr
```

FIGURE 4C
Page 2 of 3

14/42

```
GAT GGG CGG GAT GAA AGC CTC TTT GAC AAC TTC TCT TCT GTT GCT CAG AGG CTC
Asp Gly Arg Asp Glu Ser Leu Phe Asp Asn Phe Ser Ser Val Ala Gln Arg Leu

GGT GTT TAC ACT GCC AAA GAC TAT GCG GAC ATT CTT GAG TTT GGG AGG
Gly Val Tyr Thr Ala Lys Asp Tyr Ala Asp Ile Leu Glu Phe Leu Val Gly Arg

TGG AAG ATT GAG AGC TTG ACC GGG CTT TCA GGT GAA GGA AAC AAA GCG CAA GAG
Trp Lys Ile Glu Ser Leu Thr Gly Leu Ser Gly Glu Gly Asn Lys Ala Gln Glu

TAC TTG TGT GGG TTG ACT CCA AGA ATC AGG AGG TTG GAT GAG GCT CAA GCA
Tyr Leu Cys Gly Leu Thr Pro Arg Ile Arg Arg Leu Asp Glu Ala Gln Ala

AGA GCC AAG AAA GGA CCC AAG GTT CCT TTC AGC TGG ATA CAT GAC AGA GAA GTG
Arg Ala Lys Lys Gly Pro Lys Val Pro Phe Ser Trp Ile His Asp Arg Glu Val

CAG CTC TAA AAAGGAA CAAAGCTATG AAACCTTTTC ACTCTCCGTC GTCCCTCATT TGATCTATCT
Gln Leu *

GCTCTTGAAA TTGGTGTAGA TTACTATGGT TTGTGATATT GTTCGTGGGT CTAGTTACAA AGTTGAGAAG

CAGTGATTTA GTAGCTTTGT TGTTTCCAGT CTTTAAATGT TTTTGTGTTT GGTCCTTTTA GTAAACTTGT

TGTAGTTAAA TCAGTTGAAC TGTTTGGTCT GT
```

FIGURE 4C
Page 3 of 3

15/42

```
GAT GCC AAA ANG CCT CAC ATG CCT CCT AGA GAA GCT CAT GTG CAA AAG   48
Asp Ala Lys Xaa Pro His MET Pro Pro Arg Glu Ala His Val Gln Lys
1                5                 10                15

ACC CAT TCA ATK CCG CCT CAA AAG ATT GAG ATT TTC AAA TCC TTG GAG   96
Thr His Ser Xaa Pro Pro Gln Lys Ile Glu Ile Phe Lys Ser Leu Glu
                 20                25                30

GGT TGG GCT GAG GAG AAT GTC TTG CAT CTT AAA CCT GTG GAG AA       143
Gly Trp Ala Glu Glu Asn Val Leu Val His Leu Lys Pro Val Glu
35                40                45
```

FIGURE 5

Amino Acid Sequence From Fragment F2

K	E	I	P	D	D	Y	FVVLVGDMITEEALPTY	Q	T	M	L	N	T
AAA	GAA	AUU	CCN	GAU	GAU	UAU		CAA	ACN	AUG	CUN	AAU	AC/N
G	G	C		C	C	C		G				C	C
			A										

Forward Primers:

5'GCTAAGCTT AAP GAP ATQ CCA GAQ GAQ TA3'　Desat 13-1
　　　　　　　　　　　　A CCG　　　　　　　Desat 13-2
　　　　　　　　　　　　　CCC　　　　　　　Desat 13-3
　　　　　　　　　　　　　CCT　　　　　　　Desat 13-4

Reverse Primers:
(complements)

3' GTQ TGN TAC GAN TTP TGCTTAAGCGA 5'　Desat 13-5a
　　　　　　　　　　　　AAQ　　　　　　　Desat 13-6a

Oligonucleotides

P = A or G
Q = T or C
N = A, C, T or C

FIGURE 6

17/42
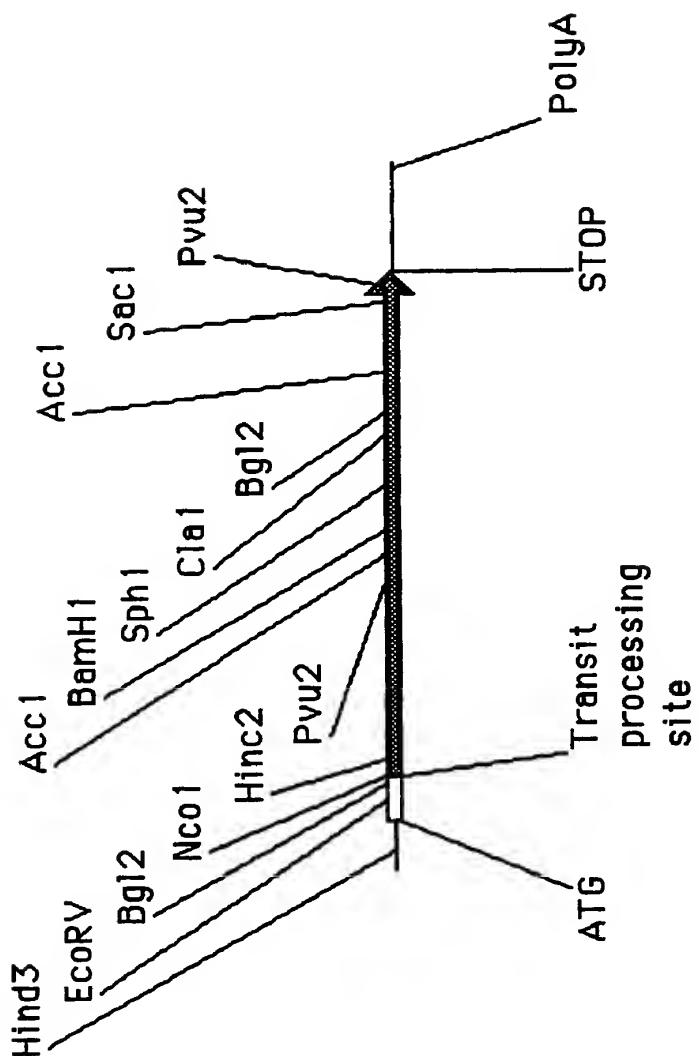
FIGURE 7A
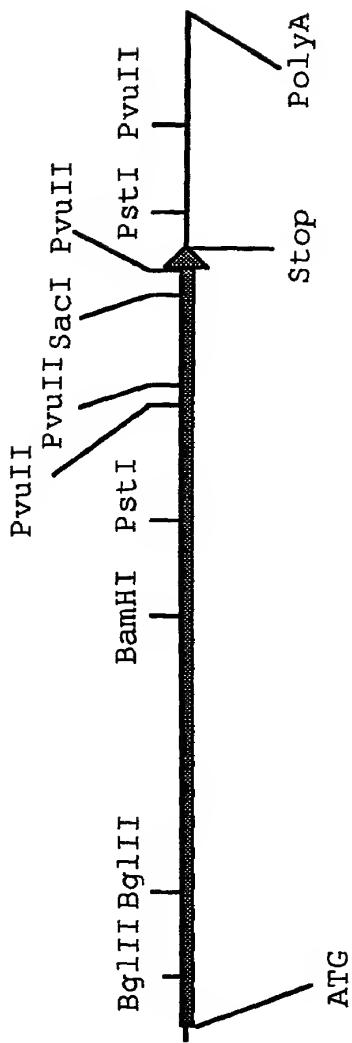
FIGURE 7B

19/42
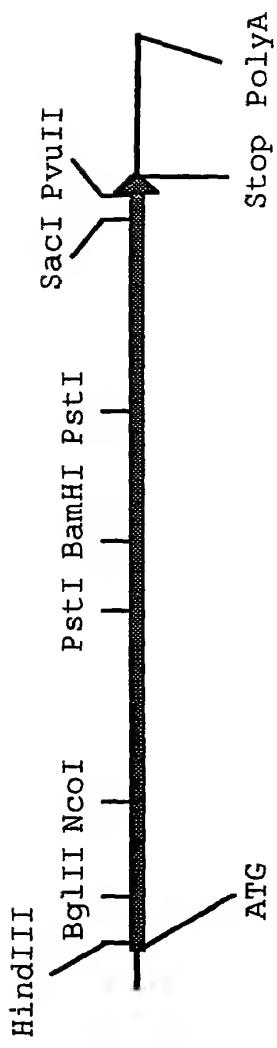
FIGURE 7C

```
TCTAGAATTC TCTAATTACG TCTGTTTGTT CTATTTTTTA TATGATATCA AATATTCGTC ATAAATATAT    70

GGTTTAAGAT GCCAAAAAAT TATTTACTTG GTGAATATAA TACGTTAAAT ATTAGAAATA CATCATTTAG   140

TTAAATAAAT AACCAAAAAC CAAAAAATTCA TATCCGCGCT GGCGCGCGGT CAGGGTCTCG TTAGTTTTAA   210

AATCAATGCA GTTACAATT  AATTCCAGC  TGAAAATAAG TATAATTTGT ATTGAAATTA TAAAGTGACA   280

TTTTTGTGT  AACAAATATT TTGTGTAACA AGAATTAAAA AAAAAAACAG AAAATACTCA GCTTTTTTAA   350

TAATAAAAAA AATTAATTGA GTTAGAAAAT TGTTGTACCA ATAACAAAAG ATTTATATGG AATTATAAAA   420

TCAACACACC AATAACACAA GACTTTTTAA AAATTTAAGA ATAATATAAG CAATAACAAT AGAATCTTCA   490

AATTCTTCAA ATCCTTAAAA ATCAATCTCC CACTATTAAT CCCCCTTAGT TTTAGTTGGT AATGGCAACG   560

TTTGTTGACT ACCGTATTGT AAATTGTCAT AAATACGTGT CAAACTCTGG TAAAAAATTA             630

GTCTGCTACA TCTGTCTTTT ATTTATAAAA CACAGCTGTT AATCAGAATT TGGTTTATTA AATCAACAAC   700

CTGCACGAAA CTTGTGTGAG CATATTTTGT CTGTTTCTGG TTCATGACCT TCTTCCGCAT GATGGCCAAG   770

TGTAATGGCC ACTTGCAAGA GCGTTTCTTC AACGAGATAA GTCGAACAAA TATTGTCCG  TTACGACCAC   840

ATATAAAATC TCCCCATCTC TATATATAAT ACCATCATGA ATACCTCAAA TCCCAATCTC             910

ACAAATACTT CAATAAAAAG ACCAAAAAAA ATTAAAGCAA AGAAAAGCCT TCTTGTGCAC AAAAAAAAAA   980
```

FIGURE 8
Page 1 of 4

```
GAAGCCTTCT AGGTTTTCAC GAC ATG AAG TTC ACT ACT CTA ATG GTC ATC ACA TTG      1036
                      MET Lys Phe Thr Thr Leu MET Val Ile Thr Leu

GTG ATA ATC GCC ATC TCG TCT CCT GTT CCA ATT AGA GCA ACC ACG GTT GAA AGT    1090
Val Ile Ile Ala Ile Ser Ser Pro Val Pro Ile Arg Ala Thr Thr Val Glu Ser

TTC GGA GAA GTG GCA CAA TCG TGT GTT GTG ACA GAA CTC GCC CCA TGC TTA CCA    1144
Phe Gly Glu Val Ala Gln Ser Cys Val Val Thr Glu Leu Ala Pro Cys Leu Pro

GCA ATG ACC ACG GGA GAC GAC CCG ACT ACA GAA TGC TGC GAC AAA CTG GTA GAG    1198
Ala MET Thr Thr Ala Gly Asp Asp Pro Thr Thr Glu Cys Cys Asp Lys Val Glu

CAG AAA CCA TGT CTT TGT GGT TAT ATT CGA TAT AGT ATG TAT GTT               1252
Gln Lys Pro Cys Leu Cys Gly Tyr Ile Arg Tyr Ser MET Tyr Val

ACT TCT CCA AAC GGT CGC AAA GTC TTA GAT TTT TGT AAG GTT CCT TTT CCT AGT    1306
Thr Ser Pro Asn Gly Arg Lys Val Leu Asp Phe Cys Lys Val Pro Phe Pro Ser

TGT TAAATCTCTC AAGACATTGC TAAGAAAAAT ATTATTAAAA ATAAAAGAAT CAAACTAGAT     1369
Cys

CTGATGTAAC AATGAATCAT CATGTTATGG TTGAAGCTTA TATGCTGAAG TGTTTGATTT TATATATGTG   1439

TGTGTGTGTG TCCTGCTCAA TTTTTGAAAC ACACACGTTT CTCCTGATTT GGATTTAAAT TATATTTTGA   1509

GTTAAAAAAAA AGAAAAAGAT GGAATGCTAT TTATACAAGT TGATGAAAAAA GTGGAAGTAC AATTTAGATA   1579
```

FIGURE 8
Page 2 of 4

```
TCTCCTACAC TTAAAGAATG AAACAATAAT AGACTTACGA AACAAATGAA AAATACATAA ATTGTCGACA  1649

ATCAACGTCC GATGACGAGT TTATTATTAA AAATTTGTGT GAAGGACTAG CAGTTCAACC AAATGATATT  1719

GAACATATAC ATCAACAAAT ATGATAAATCA TAAAAGAGAG AATGGGGGGG GGGTGTCGTT TACCAGAAAC  1789

CTCTTTTTCT CAGCTCGCTA AAACCCTACC ACTAGAGACC TAGCTCTGAC CGTCGGCTCA TCGGTGCCGG  1859

AGGTGTAACC TTTCTTTCCC ATGACCCGAA ACCTCTCTTT CCCAACTCAC GAAAACCCTA CAATCAAAAA  1929

CCTAGCTCCG ACCGTCGGCT CATCGGTGCC GAAGGTGTAA CCTTTCTCTC CCATCATAGT TTCTCGTAAA  1999

TGAAAGCTAA TTGGGCAATC GATTTTTTAA TGTTTAAACC ATGCCAAGCC ATTTCTTATA GGACAATTGT  2069

CAATAATAGC ATCTTTTGAG TTTGTCTCA AAAGTGACAC TAGAAGAAAA AAGTCACAAA AATGACATTC  2139

ATTAAAAAGT AAAATATCCC TAATACCTTT GGTTTAAATT AAATAAGTAA ACAAAAATAA ATAAAAACAA  2209

ATAAAATAAA AATAAAAAAT GAAAAAAAGA AATTTTTTTA TAGTTTCAGA TTATATGTTT TCAGATTCGA  2279

AATTTTTTAA ATTCCCTTTT TTAAATTTTC TTTTTTTTTT TGAAAATTTT TGAAACTGTT  2349

TTTAAAATTT TTATTTTTAA TTTTTTAGTA TTTATTTTTT ATTTTATAAA ATTTTAAACG CTAATTCCAA  2419

AACTCCCCCC CCCCCCCCCC CCCCAATTCT CTCCTAGTCT TTTTCTCTTT CTTATATTTG GGCTTCTATC  2489

TTCTCTTTTT TTTTCAGGCC CAAAGTATCA TGTGTAACAA CCGGTGTTCA AAAACGCGCC CGCCTGGCCG  2559
```

FIGURE 8
Page 3 of 4

```
TTTACTCGCC CGATTAAATG ATGATCGGAA GGCTGCCATG GCGAGGCGGA GGTAATCAGT GGTTCTAGGC   2629
GCTGAAACTA GAAAACCTTC AAAAATCGAA ATTTTAAGAG CTAAATCGGT GTTTATCTCA TGAATCTATT   2699
ATATTTAGTT GAAACTCACA AGAATCGGTT GTAAAAACTA TGAAATCGTG CAAAAAAAAT GAAGAACAAA   2769
ATATTCTCAG ATCTGGAAAA CACAGAGAAG AGGTTGAAGA TGAGGGTAAA ATCGTATTTT GTCATTCATT   2839
AAACTAAAAT CAAAAAAAAA TGATGCAAAA TTCAATGATA ATAACTCGAA CTCGCAACCA TATGCATCTT   2909
TAGACTGCGA CACGGACCAC TAGACTAAGC AATTTTAATG TTTATTCATC ACAGACCTAA TATATGTCTA   2979
AAACTAGGCG CCGAGTACGC CCCGCTTAAT CCCGAGTTTT TGTTAGCTCG CTAGACCCAG GGTCACCGCC   3049
CGACTAACGA GTAGCGTAAT TCTGAACTGG GGTAACAACA TAGAGAACAT CGCCGACCCT TCCCTGCCGA   3119
TGATGCCGCC TCCGATGAAC TTCCTGTAAC GCCTTCAGTT TCCATTGATT TTCCCCTTTA ATCTGATCAG   3189
TTCCATGTTT TATCCAACTC ATCCCACTCC GTAGCATTTA ATCGATCTCA TCATTACAT  ACATAACCAG   3259
TAGGAGGTCT CATATAAATT TGAACGTTTC CAGCGATGAA CAGTGCCAAT CTCTGCCGAAA TCCATTTCTC   3329
TAAGCTCAGG GCTGGCGGCT GCAGCCCGGG GATCCACTAG TTCTAGGCGG CCGCACCGCG GTGGAGCTCC   3399
AATTCGCCCT ATAGTGAGTC GTATTACGCG CGCTCACTGG C                                  3440
```

FIGURE 8
Page 4 of 4

XhoI
|
1 CTCGAGAGCTGAAGGATTTTTGTTAGAGATTCAACGACAGATGGACCCTTCCTCCACTAGGCAACTGC 69
2

70 AAGAACCTAACAATGCAAATATCACTCCTCCTCAGCCTTCAAGGAGCGTTAATAGGACTGGAACAAGCG 138

Bg1II
|
139 GTCAAGTGAGTAAATTTCCTTCCAAGATAGATCTCTATGGTTCGGTTCATGAAGTTTGTGGTTTAATT 207
169

208 GTGTAGCAACAGGGATAGTGCAAGTGAGAATAGAGTTCGACCTCATCTCACCTACCCCGGAACCTCTGAAT 276

277 GTATCCCCATTGAAGAAGAGAGGGCAAATCCTGCACCCCAGAAGGATAAAGAAATTTTGGACGCCTGAA 345

346 GAAGTGGCAGTTCTGAGGGAAGGAGTAAAAGAGTATGTCTACTACTACTCTATAATCAAGTTTCAA 414

415 GAAGCTGAGCTGGCTCTCACTTTATATGTTGATGTTGTTGTGCAGGTATGGTAAATCATGGAAAGAG 483

484 ATAAAGAATGCAAACCCTGAGTATTGGCAGAGTATTGGCAGAGAGGACTGAGGTGAGAGAGCATGTCACTTTTGTGTTA 552

553 CTCATCTGAATTATCTTATATGCGAATTGTAAGTGGTACTAAAAGGTTTGTAACTTTTGGTAGGTGGAT 621

622 TTGAAGGATAAATGGAGGAACTTGCTTCGGTAGCCGGTAACAAGTTTTATATTGCTATGAAGTTTTTTTG 690

691 CCTGCGTGACGTATCAGCAGCTGTGGAGAAGATGGTATTAGAAAGGGTCTTTTCACATTTGTGTTGTG 759

FIGURE 9
Page 1 of 6

760 ACAAATATTAATTCGGCCGGGTATGGTTTGGTTAAGACTTGTTGTTGAGAGACGTGTGGGGTTTTTGATGTA 828

829 TAATTAGTCTGTGTTTAGAACGAAACAAGACTTGTTGCGTATGCTTTTTTTAACTTGAGGGGGTTTGTT 897

Bgl II
|

898 GTTGTTAGTTAGGAACTTGACTTGTGTCTCTTTCTCTCAAGATCTGATTGGTAAGGTCTGGGTGGTAGTA 966
937

967 CTGTTTGGTTTAATTTGTTTGTTTGACTATTGAGTCACTGTGGCCCATTGACTTTAAATTAGGCTGGTATAT 1035

1036 TTTTTGGTTTAAAACCGGTCTGAGATAGTGCAATTTCGATTCAGTCAATTTTAAATTCTTCAAGGTAAT 1104

1105 GGGCTGAATACTTGTATAGTTTTAAGACTTAACAGGCCCTTAAAAGGCCCATGTTATCATAAAACGTCAT 1173

HindIII
|

1174 TGTTTAGAGTGCACCAAGCTTATAAAATGTAGCCAGGCCCTTAAAAGACTTAACAGGCCCTTAAAAGACTT 1242
1190

1243 AACATTCCTTAAAAGGCCCATGTTATCATAAAACGTCATCGTTTTGAGTGCACCAAGCTAAATGTAGCC 1311

1312 AGGCCCTTAAAAGACTTAACAGGCCCTTAAAAGGCCCATGTTATCATAAAACGCCGTCGTTTTGAGTGCAC 1380

FIGURE 9
Page 2 of 6

26/42

```
      HindIII
       |
1381  CAAGCTTATAAATGTAGCCAGCTACCTCGGGACATCACGCTCTCTTTGTACACTCCGGCCATCTCTCTCT  1449
       1383

       XhoI   BglII                                                   SalI
        |      |                                                        |
1450  CTCGAGCAGATCTCTCTCGGGAATATCGACAATGTCGACCACTTTCTGCTCTTCCGTCTCCATGCAAGC  1518
       1451  1458                                                     1484

1519  CACTTCTCTGGTAATCTCATCTCCTCTTGTGTTCCCAGATCGCTCTCGATCATACTTTCTTTAGATCA  1587

1588  TTTGCCTCTGATCTGTTGCTTGATGTTTGTTAACTCTCCACGCATGTTGATTATGTTGAGAATTAGAA  1656

1657  AAAAAATGTTAGCTTTACGAATCTTTAGTGATCATTCAATTGGATTTGCAATCTTGTGTGACATTTGA  1725

1726  GGCTTGTGTAGATTTCGATCTGTATTCATTTTGAATCACAGCTATAATAGTCATTTGAGTAGTAGTGTT  1794

1795  TTTAAATGAACATGTTTTGTTTGTATTGATGGAACAAACAGGCAGCAACAACGAGGATTAGTTTCCAGAA  1863

1864  GCCAGCTTTGGTTTCAACGACTAATCTCTCCTTCAACCTCCGCGTTCAATCCCCACTCGTTTCTCAAT  1932

1933  CTCCTGCGCGGTATGTTCTCATTCTCCAGCATTTATTCGAGCTTGTGCTTGTCATGGTACTCTCTAATT  2001

2002  GTCTATTTGGTTTATTAGGCCAAACCAGAGACGGTTGAGAAAGTGTCTAAGATAGTTAAGAAGCAGCTA  2070
```

FIGURE 9
Page 3 of 6

```
2071 TCACTCAAAGACGACCACCAAAAGGTCGTTGCGGAGACCAAGTTTGCTGATCTTGGAGCAGATTCTCTCGAC 2139

2140 ACTGTAAGTCATCAATCATTCTCTTATGTGAATAAAGAGAACTTGAAGAGTTTGTTTTTAACATATTAA 2208

2209 CTGAGTGTTTTGCATGCGCAGGTTGAGATAGTGATGGGTTTAGAGGAAGAGTTTGATATCGAAATGGCTGA 2277
                                                             EcoRV
                                                               |
                                                             2264

2278 AGAGAAAGCTCAGAAGATTGCTACTGTGGAGGAAGCTGCTGAACTCATTGAAGAGCTCGTTCAACTTAA 2346
                                                     SstI
                                                       |
                                                     2335

2347 GAAGTAATTTAGTATTAAGAGCAGCCAAGGCTTTGTGGGTTGTGTTTCATAATCTTCCTGTCAT 2415

2416 TTTCTTTTTCTTTAATGTGTCAAGCGACTCTGTTGGTTAAAGTAGTATCTGTTGCCATGGATCTCTC 2484

2485 TCTATTTGTCGACTGAAAACTTTTGGTTTACACATGAAAGCTTGTTCTCTTGTTCTTTCTTAAATCGAAAT 2553
              SalI                         HindIII
                |                             |
              2493                          2523

2554 GCCAAATGCGAGATTAGGGAATCTTGTATTAACACATACATAAGTCAAAGAGTAGGCCCTAAGATGACA 2622

2623 ATTTATAAACAATCCTATTCACATTGTATATACACAGGTTATGATTATTCCCAATCAGCGGTCAAAGAATCC 2691
```

FIGURE 9
Page 4 of 6

2692 AGCATCTTTCATCTCTGAATAGTAGACATTCTTCCTCCTGCACCAAAAACCAGTA 2760

2761 CTAAAATCATGAACATTGCAATAATCACATGCCTAGGCGGAGAGTTTGGTGATGTGGTTAGTGATAGT 2829

2830 GATACTGATGGTGCTAGAGCGGTTAAGAAGGATTAACCTGGAAGAAGTCTGCAAGGAAAGTAACATAGA 2898

2899 GAAGAGGAAGATAGGAGTGGTAACAAACACTTGTGATCCCATACAGCCTCCCAGCATTTTCAAATGTT 2967

2968 ATTTCCTTACATAAAGAAACAAGAGAAGTCTGACTAGATGATATTTATATAGGATAAGTGTTTTACCAT 3036

3037 AAGCCAAAGTGAGCGCCGTTGCAAGAGCTAACCAGACAGTACACGTTGGCATATATCTCATCAACAT 3105

3106 GATCTGAAAAGTAACATATCACAGTTAATGAACACAATGGTTACCTTGAGAAGCAAATCAAGACCTATA 3174

3175 ACAAGCCCAGAGATGAGGAAAGTCCGTGTCAACGCTTCACCGCCATTCGCGTAGTTTCCTTGGAAGACA 3243

3244 AAGGCCACCAACCAAACTTACTTCCAGAAACAACACTCCAAATGTGTCAACAAAGTCAATAGATTCCA 3312

3313 AACTACTTCGTTACAGGGTTGTATAGATAATAATATATAGAATAGTGGGAAGATAGTATAAATAAAATAAA 3381

3382 TAAAAGATCCTATCGGTAAATAGTTTATAATATCGGGGGCGTATATAAAGTATAAAAGAAACTCTTCTC 3450

3451 CAATCCGACCGTTGAAAATCACTCTCAATCTCTGGCGTAACGACCGGATCGTTCGCGCGCGTAATTTTCGC 3519

3520 TGCTATAAATAGAAACTTTCCCTCTTCTGTTTCTCGATCAAAATTTTTTTTTGGAAAAATTAAGTTTGAA 3588

FIGURE 9
Page 5 of 6

```
3589  TCTATCGTAGATGCTGTGTGACAAAAAAAAATTGTTTATCGAAGATGAGAAACATGAGGCCTGTTCATGC  3657

                      BamHI
                        |
3658  AAGGAACCAGACCACGGATCCATCTTCGCCGATGATGACGTCTCCTCTGATGAATCGTCACGCACGGAC  3726
                     3674

             BamHI
               |
3727  AGGATCCAACGCTGGACCAGCATCTAACGCCAAGAAAGCACAGACGAAAGCAGCTCAGAGACTCGC  3795
           3729

3796  GGCTGTGATGTCGAACCAAACAGGCGACGATGAAGAGACAGTGATGATGACCTTTCCTTTGACTACAAACGC  3864

                       BglII
                         |
3865  TGTCGGAAGCATTGGTCTCGCTGCCGGAAGATCT  3898
                                  3894
```

FIGURE 9
Page 6 of 6

Lambda CGN1-2

NCG-186 Linear   LENGTH = 4325

```
                         XhoI
                         AvaI
                         |
  1 CTCGAGGCAGTCACTAACATGAAGTTTGACGAGGAGCCCAACTATGGGAAGCTTATTTCTCTTTTCGAT  69
    2                          36        39        50
                               |         |         |
                               NlaIV     SduI      HindIII
                               HgiJII

                               XbaI                          SacI
                               |                             |
 70 ACTCTAATTGAGCCGTGCGCTCTATCTAGACCAATTAGAATTGATGGAGCTCTAAAGGTTGCTGGCTGT  138
                 95                                       121

                 NdeI                     SspI               NdeI
                 |                         |                  |
139 TTTCTTGTTCATATGATTAACTTCTAAACTTGTGTATAAATATTCTCTGAAAGTGCTTCTTTTGGCATA  207
              150                     180                     206

                         Ksp632I
                         |
208 TGTAGGTTGGGCAAAAAACGAGGAAGATTGCTTCTCAATTGGAAGAGAGGATGAACAGCCGAAGAAGAAAA  276
                         245
```

FIGURE 10
Page 1 of 13

```
                                        XhoII
                                         |
277  TAAGAATAGGCAGTCCTGCTACTCAATGGATCTCAGTCTCTATAACGGTCGTCGTCCCATGAAACAGAGGT  345
                                      305

                                                        MmeI    EcoRV
                                                         |        |
346  AAAACATTTTTTGCATATACACTTTGAAAGTTCCTCACTAACTGTGTAATCTTTTGGTAGATATCACTA  414
                                                        401      408

                  SduI
                  MstI                                  HaeI
                  BclI HgiAI                             |
415  CAATGTCGGAGAGAGACAA3GGCTGMNCANCATATACAAAAGGGAAATGAAGAGATGGCCTTTTGATTAGCTG  483
                  | |   |                                469
                 437 439 442

                       SduI
                       HgiJII
                         |
484  TGTAGCATCAGCAGCTAATCTCTCTGGGCTCTCATCATGGATGCTGGAACTGGATTCACTTCTCAAGTTTA  552
                       512
```

FIGURE 10

Page 2 of 13

```
           Cfr10I
           BbvII
           |   |
553   TGAGTTGTCACCGGTCTTCCTACACAAGGTAATAATCAGTTGAAGCAATTAAGAATCAATTGATTTGT  621
           560
           563

622   AGTAAACTAAGAAGAACTTACCTTATGTTTCCCCGCAGGACTGGATTATGGAACAATGGGAAAGAAC  690
                                                    SacI
                                                    |
691   TACTATATAAGCTCCATAGCTCGGTTCAGATAACGGGAGCTCTTTAGTTGTTATGTCAAAAGGTTAGTGT  759
                                                    731

                      BbvII
                      |
760   TTAGTGAATAATAAACTTATACCACAAAGTCTTCATTGACTTATTTATATACTTGTTGTGAATTGCTAG  828
                      782

829   GAACTACTTATTCTCAGCAGTCATACAAAGTGAGTGACTCATTTCCGTTCAAGTGGATAAATAAGAAAT  897

898   GGAAAGAAGATTTCATGTGTAACCTCCATGACAACTGCTGGTAATCGTTGGGGTGTGGTAATGTCGAGGA  966

              BclI
              |
967   ACTCTGGCTTCTCTGATCAGGTAGGTTTTTGTCTCTTATTGTCTGGTGTTTTTATTTTCCCCTGATAGT  1035
              981
```

FIGURE 10
Page 3 of 13

1036 CTAATATGATAAACTCTGCGTTGTGAAAGGTGGTGGAGCTTGACTTTTTGTACCCAAGCGATGGGATAC 1104

1105 ATAGGAGGTGGGAGAATGGGTATAGAATAACATCAATGGCAACTGCGATCAAGCAGCTTTCATAT 1173

ScaI
|

Tth111I
|

1174 TAAGCATACCAAAGCGTAAGATGGTGGATGAAACTCAAGAGACTCTCCGCACCACCGCCTTTCCAAGTA 1242
1175   1242

XhoII
|

1243 CTCCATGTCAAGGTTGGTTTCTTTAGCTTTGAACACAGATTTGGATCTTTTTGTTTGTTTCCATATACT 1311
                                                        1285

1312 TAGGACCTGAGAGCTTTTGGTTGATTTTTTTTCAGGACAAATGGGCGAAGAATCTGTACATTGCATCA 1380

AflII
|

1381 ATATGCTATGGCAGGACAGTGTGCTGATACACACTTAAGCATCATGTGGAAAGCCAAAGACAATTGGAG 1449
                        1415

1450 CGAGACTCAGGGTCGTCATAATACCAATCAAAGACGTAAAACCAGACGCCAACCTCTTTGGTTGAATGTA 1518

SspI
|

1519 ATGAAAGGGATGTGTCTTGGTATGTATGTACGAATAACAAAAGAGAAGATGGAATTAGTAGTAGAAAATA 1587
                                                                    1587

FIGURE 10
Page 4 of 13

34/42

EcoRV
                                                                        |
                                                                       1635
1588 TTTGGGAGCTTTTTAAGCCCTTCAAGTGTGCTTTTTATCTTATTGATATCATCCATTTGCGTTGTTTAA 1656

          XbaI
           |
          1664
1657 TGCGTCTCTAGATATGTTCCTATATCTTTCTCAGTGTCTGATAAGTGAAATGTGAGAAAACCATACCAA 1725

          SspI
           |
          1734
1726 ACCAAAATATTCAAATCTTATTTTTAATAATGTTGAATCACTCGGAGTTGCCACCTTCTGTGCCAATTG 1794
                                                                       1789

                                                                        EcoRI
                                                                         |
                                                                        1859
1795 TGCTGAATCTATCACACTAGAAAAAACATTCTTCAAGGTAATGACTTGTGGACTATGTTCTGAATTC 1863

          Eco57I
           |
          1904
1864 TCATTAAGTTTTTATTTCTGAAGTTTAAGTTTTTACCTTCTGTTTGAAATATATCGTTCATAAGATG 1932

FIGURE 10
Page 5 of 13

```
                                                    SphI
                                                    NspI
                                                    ─
1933 TCACGCCAGGACATGAGCTACACATCGCACATAGCATGCAGATCAGGAGGACGATTTGTCACTCACTTCAAA 2001
                                                              1971

                                        SphI
                             NdeI   NspI            PmaCI
                             [AvaIII] SspI AflIII
                             ─     ─    ─  ─
2002 CACCTAAGAGAGCTTCTCTCTCTCACAGCGGCACACACACATATGCAATATTTACACGTGATCGCCATGCAA 2070
        Tth111II                   2037 2048 2053
        ─                          2036 2044      2056
        2015

                        SecI
                        ─
2071 ATCTCCATTCTCACCTATAAATTAGAGCCTCGGCTTCACTCTTTACTCAAACCAAAACTCATCACTACA 2139
                                            2099

                    Ksp632I
                    ─
2140 GAACATACACAAATGGCGAACAAGCTCTTCCTCGTCTCGGCAACTCTCGCCTTGTTCTTCCTTCTCACC 2208
      METAlaAsnLysLeuPheLeuValSerAlaThrLeuAlaLeuPhePheLeuLeuLeuThr
                              2171
```

FIGURE 10
Page 6 of 13

```
                              SalI
                              HindII                    NaeI
                              AccI                       Cfr10I
                    AccI       |||                        |   |
                     |
2209  AATGCCTCCGTCTACAGGACGGTTGTGGAAGTCGACGAAGATGATGCCACAAATCCAGCCGGCCCATTT  2277
      AsnAlaSerValTyrArgThrValValGluValAlaAspAspAlaThrAsnProAlaGlyProPhe
                     2220      2241           2267
                              2242            2269
                              2240

                                        Tth111I
                                        HindIII               NlaIV
                                        |                      |
2278  AGGATTCCAAAATGTAGGAAGGAGTTTCAGCAAGCACAACACCTGAAAGCTTGCCAACAATGGCTCCAC  2346
      ArgIleProLysCysArgLysGluGlnPheGlnGlnAlaGlnHisLeuLysLysAlaCysGlnTrpLeuHis
                                                     2325            2342

                  Tth111I           NlaIV                               BbvII
                   |                 |                                   |
2347  AAGCAGGCAATGCAGTCCGGTAGTGGTCCAAGCTGGACCCTCGATGGTGAGTTTGATTTGAAGACGAC  2415
      LysGlnAlaMETGlnSerGlySerGlyProSerTrpThrLeuAspGlyGluPheAspLeuLysThrThr
                  2363                          2384                      2415

              NlaIV
              ApaI GsuI HaeI NspBII                         SacI
              |     |    |    |                              Ksp632
                                                             |   |
2416  GTGGAGAACCAACAACAGGGCCCGCAGCAGGCCACCGGCTGCTCCAGCAGTGCTGCAACGAGCTCCAC  2484
      ValGluAsnGlnGlnGlnGlyProGlnGlnAlaThrGlyCysSerSerSerAlaAlaThrSerSerLeuHis
              2436 2438 2444 2449 2455                       2481
```

FIGURE 10
Page 7 of 13

```
2485 CAGGAAGAGCCACTTTGCGTTGCCCAACCTTGAAAGGAGCATCCAAAGCCGTTAAACAACAGATTCGA 2553
     GlnGluProLeuCysValCysProThrLeuLysGlyAlaSerLysAlaValLysGlnIleArg

2554 CAACAACAGGGACAACAAATGCAGGGACAGCAGATGCAGCAAGTGATTAGCCGTATCTACCAGACCGCT 2622
     GlnGlnGlnGlyGlnGlnMETGlnGlyGlnGlnMETGlnGlnValIleSerArgIleTyrGlnThrAla

                                                                    SecI
                                                                    BbvII
                                                                     |
2623 ACGGCACTTACCTAGAGCTTGCAACATCAGGCAAGTTAGCATTGCCCCTTCCAGAAGACCATGCCTGGG 2691
     ThrHisLeuProArgAlaCysAsnIleArgGlnValSerIleCysProPheGlnLysThrMETProGly
                                                                 2684
                                                                    2687

                                       XhoI    SecI
                                       AvaI    DsaI
                                        |      AccI
                                               |
2692 CCCGGCTTCTACTAGATTCCAAACGAATATCCTCGAGAGTGTGTATACCACGGTGATATGAGTGTGGTT 2760
     ProGlyPheTyr .
                              2724    2736
     2694                                  2740
     2692

          HpaI
          HindII
          |
2761 GTTGATGTGTATGTTAACACTACACATAGTCATGGTGTGTGTTCCATAAATAATGTACTAATGTAATAAGAAC 2829
     2774
```

NlaIV
HgiJII
ApaI
|

FIGURE 10
Page 8 of 13

38/42

```
                                                                  AccI
                                                                   |
2830 TACTCCGTAGACGGTAATAAAAGAGAGAAGTTTTTTTTTTTACTCTTGCTACTTTCCTATAAAGTGATGAT 2898
                                                                 2838

                                                                 VspI          SpeI
                                                                  |            ScaI
                                                                 2929          | |
2899 TAACAACAGATACACCCAAAAAGAAAACAATTAATCTATATTCACAATGAAGCAGTACTAGTCTATTGAA 2967
                                                                            2954
                                                                            2955

            NspI
     AflIII
       |  |                                                           XhoII
2968 CATGTCAGATTTCTTTTTCTAAATGTCTAATTAAGCCTTCAAGGCTAGTGATGATGATAAAAGATCATCCA 3036
       2968                                                             NlaIV
            2972                                                        BamHI    MmeI
                                                                          |      BclI
                                                                         3041     |
                                                                         3043   3069

3037 ATGGGATCCAACAAAGACTCAAAATCTGGTTTGATCAGATACTTCAAAACTATTTTGTATTCATTAAA 3105
```
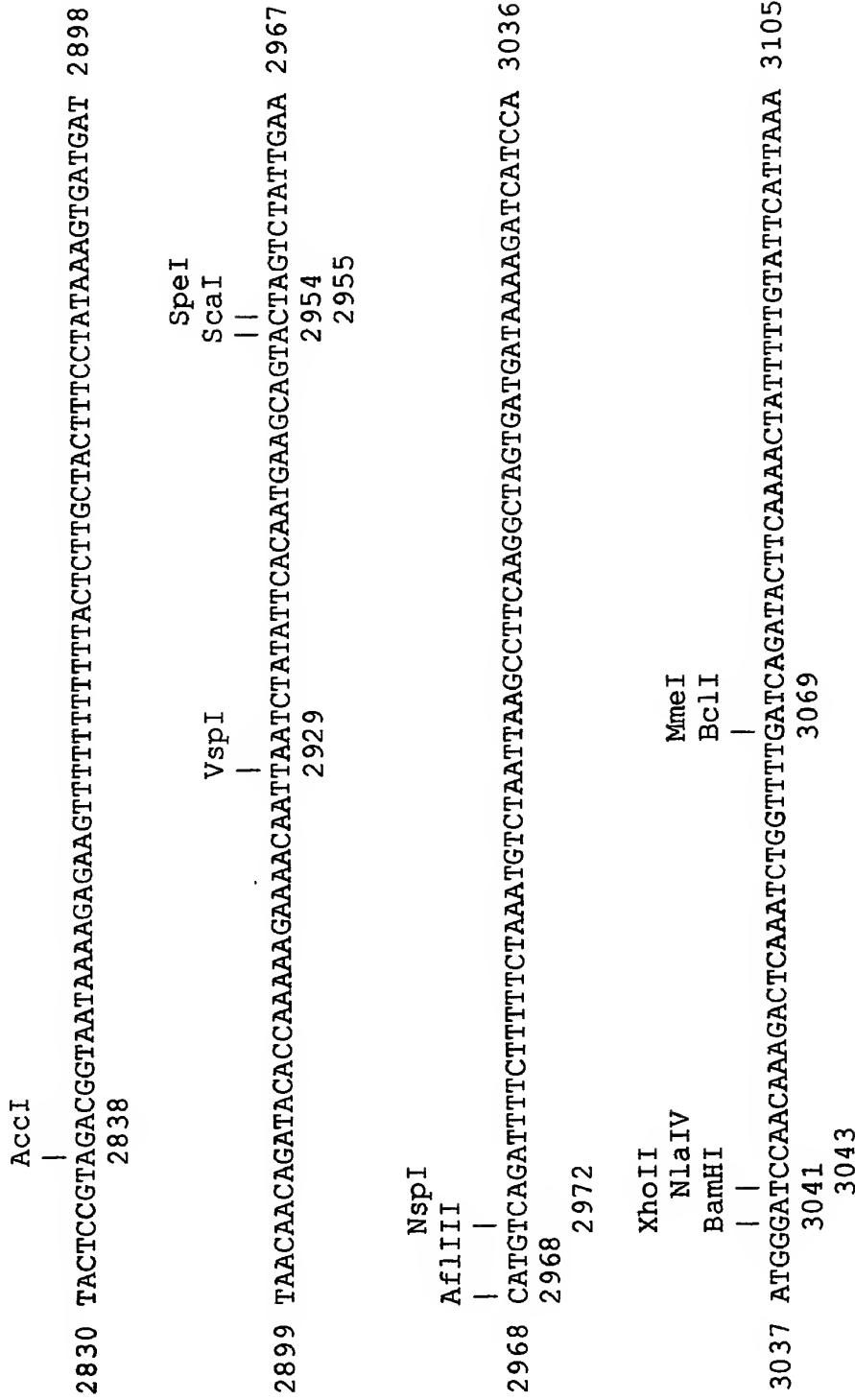
FIGURE 10
Page 9 of 13

Tth111

3106 TTATGCAAGTGTTCTTTATTTGGTGAAGACTCTTTAGAAGCAAAGAACGACAAGCAGTAATAAAAAAA 3174
BbvII   3174
                                                                           3139

VspI

3175 ACAAAGTTCAGTTTTAAGATTTGTTATTGACTTATTGTCATTTGAAAAATATAGTATGATATTAATATA 3243
                                                                           3237

VspI

3244 GTTTTATTTATATAAATGCTTGTCTATTCAAGATTTGAGAACATTAATATGATACTGTCCACATATCCAA 3312
Tth111II
3250

                                                    NdeI   Tth111II
3313 TATATTAAGTTTCATTTCTGTTCAAACATATGATAAGATGGTCAAATGATTATGAGTTTTGTTATTTAC 3381
                                                     3341   3352
                                                                           3287

                                                              Eco57I

3382 CTGAAGAAAAGATAAGTGAGCTTCGAGTTTCTGAAGGGTACGTGATCTTCATTTCTTGGCTAAAAGCGA 3450
     Eco57I
     3404   3434

3451 ATATGACATCACCTAGAGAAAGCCGATAATAGTAAACTCTGTTCTTGGTTTTTGGTTTAATCAAACCGA 3519

FIGURE 10
Page 10 of 13

```
                              Tth111I
     Cfr10I                   NdeI
       |                       ||
3520 ACCGGTAGCTGAGTGTCAAGTGTCAGCAAACATCGCAAACCATATGTCAATTCGTTAGATTCCCGGTTTAA 3588
       3521                    3560
                               3561

          Cfr10I
           |
3589 GTTGTAAACCGGTATTTCATTTGGTGAAAAACCCTAGAAGCCAGCCANCCTTTTAATCTAATTTTTGCA 3657
         3597

                                                        NlaIV
                                                        HindII
                                                        HgiCI
                                                        BspHI
                                                        |||
3658 AACGAGAAGTCACCACACCCTCTCCACTAAAAACCCTGAACCTTACTGAGAGAAGCAGAGNCANNAAAGAA 3726
                                                        3717
                                                        3716
                                                          3718

          Eco31I   PmaCI                        Ksp632I
            |        |                             |
3727 CAAATAAAACCCGAAGATGAGACCACCACGTGCGGCGGGACGTTCAGGGGACGCGGGAGGAAGAGAATGR 3795
            3740     3756                          3781

3796 CGGCGGG5MNTTTGGTGGCGGCGGCGGACGTTTGGTGGCGGCGGCGGTGGACGTTTTGGTGGCGGCGGTGGA 3864
```

FIGURE 10
Page 11 of 13

EcoRV
|
3880

3865 CCTTTGGTGGTGGATATCGTGACGAAGGACCTCCCAGTGAAGTCATTGGTTCGTTCGTTTACTCTTTTCTTAG 3933

HindIII
AflII
| 3977
| 3974

3934 TCGAATCTTATTCTTGCTCTGCTCGTTGTTTACCGATAAAGCTTAAGACTTTATTGATAAAGTTCTCA 4002

4003 GCTTTGAAATGTGAAATGAACTGTTCCTGCTTATTAGTGTTCCTTTGTTTTGAGTTGAATCACTGTCTTA 4071

Tth111II
|
4179

4072 GCACTTTTGTTAGATTCATCTTTGTGTTAAGTTAAAAGGTAGAAACTTTGTGACTTGTCTCCGTTATG 4140

HpaI
HindII
|
4149

4141 ACAAGGTTAACTTTGTGGTTATAACAGAAGTTGGCGACCTTTCTCCATGCTTGTGAGGGTGATGCTGTG 4209

XhoII
|
4231

4210 GACCAAGCTCTCTCAGGCGAAGATCCCTTACTTCAATGCCCCAATCTACTTGGAAAACAAGACACAGAT 4278

FIGURE 10
Page 12 of 13

42/42

```
                                            SalI
                                            PstI
                HindIII              HindII
           XhoII BspMI               AccI EcoRI
             |     |                  | |||   |
4279 TGGGAAAGTTGATGAGATCCAAGCTTGGGCTGCAGGTCGACGAATTC 4325
           4294  4300              4313  4316 4321
                 4302                    4317
                                    4315
```

FIGURE 10
Page 13 of 13

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/01746

I. CLASSIFICATION OF BJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 1/21, 15/29, 15/82; CO7H 15/12
U.S. CL.: 435/172.3, 240,4, 252.3; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	435/172.3, 240.4, 252.3 ; 536/27 800/205, DIG.69

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are included in the Fields Searched 8

USPTO AUTOMATED PATENT SYSTEM: DIALOG FILES BIOTECH AND PATENTS.
SEE ATTACHMENT FOR SEARCH TERMS

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
	SEE ATTACHED PAGES	

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
24 June 1991	**07 AUG 1991**
International Searching Authority	Signature of Authorized Officer
RO/US	P.Rhodes

Form PCT/ISA/210 (second sheet) (Rev.11-87)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A		
Y	World Soybean Research Conference III: Proceedings (Westview Press): Shibles (ed); Published 1985; Goodman et al; "Biotechnology and its impact on future improvements in soybean production and use"; pages 261-271. See pages 264-265.	1-22, 34-37
Y	Journal of Lipid Research; Volume 26; Issued 1985; Mattson et al; "Comparison of effects of dietary saturated, monounsaturated, and poly-unsaturated fatty acids on plasma and lipids and lipoprotein in man"; pages 194-202. See entire document.	1-22, 34-37
Y	EP A 0,255,377 (KRIDL et al) 03 February 1988. See entire document.	1-22, 34-37
Y	Trends in Biotechnology; Volume 5; Issued February 1987; Knauf; "The application of genetic engineering to oilseed crops"; pages 40-47. See entire document.	1-22, 34-37
Y	Trends in Biotechnology; Volume 7; Issued May 1989; Battey et al; "Genetic engineering for plant oils: potential and limitations"; pages 122-126. See entire document.	1-22, 34-37
Y	US, A, 4,446,235 (SEEBURG) 01 May 1984. See entire document.	1-22, 34-37
Y	US, A, 4,394,443 (WEISSMAN et al) 19 July 1983. See entire document.	1-22, 34-37
Y	Methods in Enzymology; Volume 71; Issued 1981; McKeon et al; "Stearoyl-acyl carrier protein desaturase from safflower seeds"; pages 275-281. See entire document.	1-22, 34-37
Y	Archives of Biochemistry and Biophysics; Volume 162; Issued 1974; Jaworski et al; "Fat metabolism in higher plants, properties of a soluble stearyl-acyl carrier protein desaturase from maturing Carthamus tinctorius"; pages 158-165. See entire document.	1-22, 34-37
Y	The Journal of Biological Chemistry; Volume 257, Number 20; Issued 25 October 1982; McKeon et al; "Purification and characterization of the stearoyl-acyl carrier protein desaturase and the acyl-acyl carrier protein thioesterase from maturing se ds of safflow r"; pages 12141-12147. See entire document.	1-22, 34-37

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Proceedings of the Flax Institute USA; Volume 41, Number 3; Downey et al; "Genetic control of fatty acid composition in oilseed crops"; pages 1-3. See entire document.	23-33, 38-45
X / Y	EP A,0323753 (WONG et al) 12 July 1989. See entire document.	23-29, 31-33 / 38-39, 41-45 / 30, 40
X / Y	Journal of the American Oil Chemists Society; Volume 61, Number 1; Issued January 1984; Wilcox et al; "Genetic alteration of soybean oil composition by a chemical mutagen"; pages 97-100. See entire document.	23, 25-27, 29, 31, 33, 38-45 / 30, 40
X / Y	Journal of the American Oil Chemists Society; Volume 59, Number 5; Issued May 1982; Wolf et al; "Effect of temperature on soybean seed constituents: oil, protein, moisture, fatty acids, amino acids and sugars"; pages 230-232. See entire document.	23, 25-27, 29, 31, 33, 38-45 / 30, 40
Y	Lipids; Volume 4, Number 6; Issued 1969; Inkpen et al; "Desaturation of palmitate and stearate by cell-free fractions from soybean cotyledons"; pages 539-543. See entire document.	30, 40
Y	The Journal of Biological Chemistry; Volume 241; Issued 1966; Nagai et al; "Enzymatic desaturation of stearyl acyl carrier protein"; pages 1925-1927. See entire document.	30, 40
X / Y	The Journal of Heredity; Volume 80, Number 3; Issued March 1989; Moore et al; "The inheritance of high oleic acid in peanut"; pages 252-253. See entire document.	23, 25-27, 29 / 31, 33, 38-45 / 30, 40
X / Y	Crop Science; Volume 24; Issued November-December 1984; Carver et al; "Developmental changes in acyl-composition of soybean seed selected for high oleic acid concentration"; pages 1016-1019. See entire document.	23, 25-27, 29 / 31, 33, 38-45 / 30, 40
X / Y	Bodman et al., "Soybeans and Soybean Products: Processing of edible soybean oil" published 1951 by Interscience Publishers, Inc. (N.Y.), pages 649-725, see only pages 702-709.	31 and 33 / 32